

DESCRIPTION

TRICYCLIC QUINOXALINE DERIVATIVES AS PROTEIN TYROSINE KINASE
INHIBITORS

5

RELATED APPLICATIONS

This application is related to and claims priority from
provisional application serial number 60/054,903, dated August 4,
1997, and provisional application serial number 60/259,686, dated
10 September 19, 1997, both of which are incorporated by reference
as if fully set forth herein.

INTRODUCTION

15 The present invention relates generally to organic
chemistry, biochemistry, pharmacology and medicine. More
particularly, it relates to tricyclic quinoxaline compounds, and
their physiologically acceptable salts and prodrugs, which
modulate the activity of protein tyrosine kinases ("PTKs") and,
20 therefore, are expected to exhibit a salutary effect against
disorders related to abnormal PTK activity.

BACKGROUND OF THE INVENTION

The following is offered as background information only and is not admitted to be prior art to the present invention.

5 Protein kinases, PKs, are enzymes that catalyze the phosphorylation of hydroxy groups on tyrosine, serine and threonine residues of proteins. The consequences of this seemingly simple activity are staggering; cell growth, differentiation and proliferation; i.e., virtually all aspects of
10 cell life in one way or another depend on PK activity.

Furthermore, abnormal PK activity has been related to a host of disorders, ranging from relatively non-life threatening diseases such as psoriasis to extremely virulent diseases such as glioblastoma (brain cancer).

15 The PKs can conveniently be broken down into two classes, the protein tyrosine kinases (PTKs) and the serine-threonine kinases (STKs).

One of the prime aspects of PK activity is involvement with growth factor receptors. Growth factor receptors are cell-surface
20 proteins. When bound by a growth factor ligand, growth factor receptors are converted to an active form which interacts with proteins on the inner surface of a cell membrane. This leads to phosphorylation on tyrosine residues of the receptor and other proteins and to the formation inside the cell of complexes with a

variety of cytoplasmic signaling molecules that, in turn, affect numerous cellular responses such as cell division (proliferation), cell differentiation, cell growth, expression of metabolic effects on the extracellular microenvironment, etc.

5 For a more complete discussion, see Schlessinger and Ullrich, Neuron, 9:303-391 (1992) which is incorporated by reference, including any drawings, as if fully set forth herein.

Growth factor receptors with PK activity are known as receptor tyrosine kinases ("RTKs"). They comprise a large family
10 of transmembrane receptors with diverse biological activity. At present, at least nineteen (19) distinct subfamilies of RTKs have been identified. An example of these is the subfamily designated the "HER" RTKs, which include EGFR (epithelial growth factor receptor), HER2, HER3 and HER4. These RTKs consist of an
15 extracellular glycosylated ligand binding domain, a transmembrane domain and an intracellular cytoplasmic catalytic domain that can phosphorylate tyrosine residues on proteins.

Another RTK subfamily consists of insulin receptor (IR), insulin-like growth factor I receptor (IGF-1R) and the insulin
20 receptor related receptor (IRR). IR and IGF-1R interact with insulin, IGF-I and IGF-II to form a heterotetramer of two entirely extracellular glycosylated α subunits and two β subunits which cross the cell membrane and which contain the tyrosine kinase domain.

A third RTK subfamily is referred to as the platelet derived growth factor receptor ("PDGFR") group, which includes PDGFR α , PDGFR β , CSFIR, c-kit and c-fms. These receptors consist of glycosylated extracellular domains composed of variable numbers of immunoglobulin-like loops and an intracellular domain wherein the tyrosine kinase domain is interrupted by unrelated amino acid sequences.

Another group which, because of its similarity to the PDGFR subfamily, is sometimes subsumed in the latter group is the fetus liver kinase ("flk") receptor subfamily. This group is believed to be composed of kinase insert domain-receptor fetal liver kinase-1 (KDR/FLK-1), flk-1R, flk-4 and fms-like tyrosine kinase 1 (flt-1).

One further member of the tyrosine kinase growth factor receptor family is the fibroblast growth factor ("FGF") receptor group. This group consists of four receptors, FGFR1-4, and seven ligands, FGF1-7. While not yet well characterized, it appears that the receptors consist of a glycosylated extracellular domain containing a variable number of immunoglobulin-like loops and an intracellular domain in which the PTK sequence is interrupted by regions of unrelated amino acid sequences.

A more complete listing of the known RTK subfamilies is described in Plowman et al., DN&P, 7(6):334-339 (1994) which is incorporated by reference, including any drawings, as if fully

set forth herein.

In addition to the RTKs, there also exists a family of entirely intracellular PTKs called "non-receptor tyrosine kinases" or "cellular tyrosine kinases." This latter designation, abbreviated "CTK", will be used herein. CTKs do not contain extracellular and transmembrane domains. At present, over 24 CTKs in 11 subfamilies (Src, Frk, Btk, Csk, Abl, Zap70, Fes, Fps, Fak, Jak and Ack) have been identified. The Src subfamily appear so far to be the largest group of CTKs and includes Src, Yes, Fyn, Lyn, Lck, Blk, Hck, Fgr and Yrk. For a more detailed discussion of CTKs, see Bolen, Oncogene, 8:2025-2031 (1993), which is incorporated by reference, including any drawings, as if fully set forth herein.

The serine-threonine kinases or STKs, like the CTKs, are predominantly intracellular although there are a few receptor STKs. STKs are the most common of the cytosolic kinases; i.e., kinases which perform their function in that part of the cytoplasm other than the cytoplasmic organelles and cytoskeleton.

The cytosol is the region within the cell where much of the cell's intermediary metabolic and biosynthetic activity occurs; e.g., it is in the cytosol that proteins are synthesized on ribosomes.

RTKs, CTKs and STKs have all been implicated in a host of pathogenic conditions including, significantly, cancer. Others

pathogenic conditions which have been associated with PTKs include, without limitation, psoriasis, hepatic cirrhosis, diabetes, atherosclerosis, angiogenesis, restenosis, ocular diseases, rheumatoid arthritis and other inflammatory disorders,
5 autoimmune disease and a variety of renal disorders.

With regard to cancer, two of the major hypotheses advanced to explain the excessive cellular proliferation that drives tumor development relate to functions known to be PK regulated. That is, it has been suggested that malignant cell growth results from
10 a breakdown in the mechanisms that control cell division and/or differentiation. It has been shown that the protein products of a number of proto-oncogenes are involved in the signal transduction pathways that regulate cell growth and differentiation. These protein products of proto-oncogenes
15 include the extracellular growth factors, transmembrane growth factor PTK receptors (RTKs), cytoplasmic PTKs (CTKs) and cytosolic STKs, discussed above.

In view of the apparent link between PK-related cellular activities and a number of human disorders, it is no surprise
20 that a great deal of effort is being spent in an attempt to identify ways to modulate PK activity. Some of these efforts have involved biomimetic approaches using large molecules patterned after those involved in the actual cellular processes (e.g., mutant ligands (U.S. App. No. 4,966,849); soluble

receptors and antibodies (App. No. WO 94/10202, Kendall and Thomas, Proc. Nat'l Acad. Sci., 90:10705-09 (1994), Kim, et al., Nature, 362:841-844 (1993)); RNA ligands (Jelinek, et al., Biochemistry, 33:10450-56); Takano, et al., Mol. Bio. Cell 4:358A
5 (1993); Kinsella, et al., Exp. Cell Res. 199:56-62 (1992); Wright, et al., J. Cellular Phys., 152:448-57) and tyrosine kinase inhibitors (WO 94/03427; WO 92/21660; WO 91/15495; WO 94/14808; U.S. Pat. No. 5,330,992; Mariani, et al., Proc. Am. Assoc. Cancer Res., 35:2268 (1994)).

10 More recently, attempts have been made to identify small molecules which act as PK inhibitors. For example, bis-monocyclic, bicyclic and heterocyclic aryl compounds (PCT WO 92/20642), vinylene-azaindole derivatives (PCT WO 94/14808) and 1-cyclopropyl-4-pyridylquinolones (U.S. Pat. No. 5,330,992) have
15 been described as PTK inhibitors. Styryl compounds (U.S. Pat. No. 5,217,999), styryl-substituted pyridyl compounds (U.S. Pat. No. 5,302,606), quinazoline derivatives (EP App. No. 0 566 266 A1), selenaindoles and selenides (PCT WO 94/03427), tricyclic polyhydroxylic compounds (PCT WO 92/21660) and
20 benzylphosphonic acid compounds (PCT WO 91/15495) have all been described as PTK inhibitors useful in the treatment of cancer.

SUMMARY OF THE INVENTION

Our own efforts to identify small organic molecules which modulate PK activity and which, therefore, would be expected to be useful in the treatment or prevention of disorders driven by abnormal PK activity, has led us to the discovery of a family of tricyclic quinoxaline compounds which exhibit such PK-modulating activity and which are the subject of this invention.

Thus, the present invention relates generally to tricyclic quinoxaline derivatives which modulate the activity of both receptor (RTK) and non-receptor (CTK) protein tyrosine kinases (PTKs). In addition, the present invention relates to the preparation and use of pharmacological compositions of the disclosed compounds and their physiologically acceptable salts and prodrugs in the treatment or prevention of PTK driven disorders such as, by way of example and not limitation, cancer, diabetes, hepatic cirrhosis, atherosclerosis, angiogenesis and renal disease.

As used herein a "tricyclic quinoxaline derivative" refers to a chemical compound having the general structure shown in Formula 1.

A "pharmacological composition" refers to a mixture of one or more of the compounds described herein, or physiologically acceptable salts or prodrugs thereof, with other chemical components such as physiologically acceptable carriers and

excipients. The purpose of a pharmacological composition is to facilitate administration of a compound to an organism.

A "prodrug" refers to an agent which is converted into the parent drug in vivo. Prodrugs are often useful because, in some
5 situations, they may be easier to administer than the parent drug. They may, for instance, be bioavailable by oral administration whereas the parent drug is not. The prodrug may also have improved solubility compared to the parent drug in pharmacological compositions. An example, without limitation, of
10 a prodrug would be a compound of the present invention which is administered as an ester (the "prodrug") to facilitate transmittal across a cell membrane where water solubility is not beneficial, but which then is metabolically hydrolyzed to the carboxylic acid once inside the cell where water solubility is
15 beneficial.

As used herein, an "ester" is a carboxy group, as defined herein, wherein R" is any of the listed groups other than hydrogen.

As used herein, a "physiologically acceptable carrier"
20 refers to a carrier or diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound.

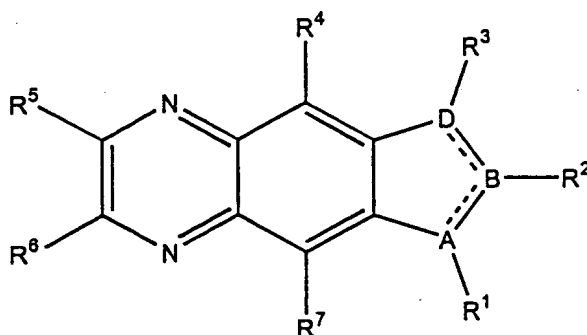
An "excipient" refers to an inert substance added to a pharmacological composition to further facilitate administration

of a compound. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

1. THE COMPOUNDS

A. General Structural Features.

In one aspect, the present invention relates to compounds having the structure shown in Formula 1:



1

The dotted line in the five-member ring containing A, B and D means that either the A-B bond or the B-D bond is a double bond.

A, B, and D are independently selected from the group consisting of carbon, nitrogen, oxygen and sulfur, such that the resulting fused 5-member ring/6-member ring is one known in the chemical arts. It is understood that when A, B or D is oxygen or sulfur, R¹, R² and R³, respectively, do not exist. Furthermore, when A, B or D is nitrogen and that nitrogen is participating in

a double bond, R^1 , R^2 or R^3 , respectively, does not exist.

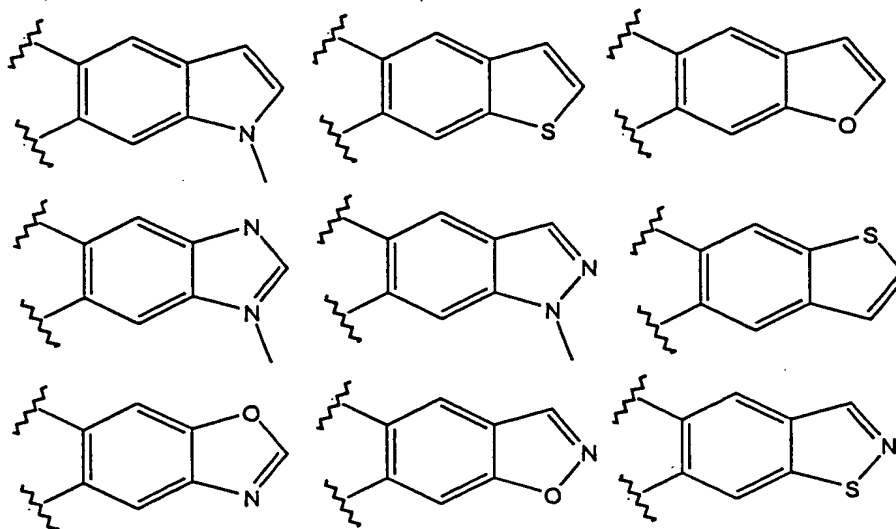
When A, B or D is nitrogen and that nitrogen is not participating in a double bond, R^1 , R^2 or R^3 is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, hydroxy, alkoxy, C-carboxy, O-carboxy, carbonyl, thiocarbonyl, C-amido, guanyl, sulfonyl and trihalomethylsulfonyl.

R^4 , R^5 , R^6 and R^7 are independently selected from the group consisting of hydrogen, alkyl, trihaloalkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy, thiohydroxy, thioalkoxy, thioaryloxy, sulfinyl, sulfonyl, N-sulfonamido, S-sulfonamido, trihalomethane-sulfonamido, carbonyl, thiocarbonyl, C-carboxy, O-carboxy, C-amido, N-amido, cyano, nitro, halo, O-carbamyl, N-carbamyl, thiocarbamyl, N-thiocarbamyl, ureido, guanyl, guanidino, amino, and $-NR^{10}R^{11}$, with the proviso that, when one of R^5 or R^6 is hydrogen, methyl or phenyl, the other is not any of hydrogen, methyl or phenyl.

R^{10} and R^{11} are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, aryl, carbonyl, sulfonyl and, combined, a five- or six-member heteroalicyclic ring.

Physiologically acceptable salts and prodrugs of the claimed compounds are also within the scope of this invention.

Examples of a "fused 5-member ring/6-member ring known in the chemical arts" include, but are not limited to:



5 As used herein, to "participate in a double bond" means to be one of two atoms which are double-bonded to one another.

As used herein, the term "alkyl" refers to a saturated aliphatic hydrocarbon including straight chain and branched chain groups. Preferably, the alkyl group has 1 to 20 carbon atoms
10 (whenever a numerical range; e.g. "1-20", is stated herein, it means that the group, in this case the alkyl group, may contain 1 carbon atom, 2 carbon atoms, 3 carbon atoms, etc. up to and including 20 carbon atoms). More preferably, it is a medium size alkyl having 1 to 10 carbon atoms. Most preferably, it is a
15 lower alkyl having 1 to 4 carbon atoms. The alkyl group may be

substituted or unsubstituted. When substituted, the substituent group is preferably one or more independently selected from cycloalkyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy, thiohydroxy, thioalkoxy, thioaryloxy, cyano, halo, carbonyl, thiocarbonyl, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, C-carboxy, O-carboxy, nitro, sulfonamido, trihalomethanesulfonamido, silyl, guanyl, guanidino, ureido, amino and $NR^{10}R^{11}$, wherein R^{10} and R^{11} are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, aryl, carbonyl, sulfonyl, trihalomethylsulfonyl and, combined, a five- or six-member heteroalicyclic ring.

A "cycloalkyl" group refers to an all-carbon monocyclic or fused ring (i.e., rings which share an adjacent pair of carbon atoms) group wherein one of more of the rings does not have a completely conjugated pi-electron system. Examples, without limitation, of cycloalkyl groups are cyclopropane, cyclobutane, cyclopentane, cyclopentene, cyclohexane, cyclohexadiene, cycloheptane, cycloheptatriene and adamantane. A cycloalkyl group may be substituted or unsubstituted. When substituted, the substituent group(s) is preferably one or more independently selected from alkyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy, thiohydroxy, thioalkoxy, thioaryloxy, cyano, halo, carbonyl, thiocarbonyl, C-carboxy, O-carboxy, O-carbamyl, N-carbamyl, C-amido, N-amido, nitro, amino and $NR^{10}R^{11}$, with R^{10}

and R¹¹ being as defined above.

An "alkenyl" group refers to an alkyl group which consists of at least two carbon atoms and at least one carbon-carbon double bond.

5 An "alkynyl" group refers to an alkyl group which consists of at least two carbon atoms and at least one carbon-carbon triple bond.

10 An "aryl" group refers to an all-carbon monocyclic or fused-ring polycyclic (i.e., rings which share adjacent pairs of carbon atoms) groups having a completely conjugated pi-electron system. Examples, without limitation, of aryl groups are phenyl, naphthalenyl and anthracenyl. The aryl group may be substituted or unsubstituted. When substituted, the substituent group is preferably one or more selected from halo, trihalomethyl, alkyl, 15 hydroxy, alkoxy, aryloxy, thiohydroxy, thioalkoxy, thioaryloxy, cyano, nitro, azido, carbonyl, thiocarbonyl, C-carboxy, O-carboxy, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, sulfinyl, sulfonyl, amino and NR¹⁰R¹¹ wherein R¹⁰ and R¹¹ are previously defined herein.

20 As used herein, a "heteroaryl" group refers to a monocyclic or fused ring (i.e., rings which share an adjacent pair of atoms) group having in the ring(s) one or more atoms selected from the group consisting of nitrogen, oxygen and sulfur and, in addition, having a completely conjugated pi-electron system. Examples,

without limitation, of heteroaryl groups are pyrrole, furan, thiophene, imidazole, oxazole, thiazole, pyrazole, pyridine, pyrimidine, quinoline, isoquinoline and purine. The heteroaryl group may be substituted or unsubstituted. When substituted, the substituted group is preferably one or more selected from alkyl, cycloalkyl, halo, trihalomethyl, hydroxy, alkoxy, aryloxy, thiohydroxy, thioalkoxy, thioaryloxy, cyano, azido, nitro, carbonyl, thiocarbonyl, sulfonamido, C-carboxy, O-carboxy, sulfinyl, sulfonyl, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, amino and $NR^{10}R^{11}$ where R^{10} and R^{11} are previously defined herein.

A "heteroalicyclic" group refers to a monocyclic or fused ring group having in the ring(s) one or more atoms selected from the group consisting of nitrogen, oxygen and sulfur. The rings may also have one or more double bonds. However, the rings do not have a completely conjugated pi-electron system. The heteroalicyclic ring may be substituted or unsubstituted. When substituted, the substituted group(s) is preferably one or more selected from alkyl, cycloalkyl, aryl, heteroaryl, halo, trihalomethyl, hydroxy, alkoxy, aryloxy, thiohydroxy, thioalkoxy, thioaryloxy, cyano, nitro, carbonyl, thiocarbonyl, C-carboxy, O-carboxy, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, sulfinyl, sulfonyl, C-amido, N-amido, amino and $NR^{10}R^{11}$ where R^{10} and R^{11} are previously defined herein.

A "hydroxy" group refers to an -OH group.

An "azido" group refers to a $-N\equiv N$ group.

An "alkoxy" group refers to both an -O-alkyl and an -O-cycloalkyl group, as defined herein.

5 An "aryloxy" group refers to both an -O-aryl and an -O-heteroaryl group, as defined herein.

A "thiohydroxy" group refers to an -SH group.

A "thioalkoxy" group refers to both an S-alkyl and an -S-cycloalkyl group, as defined herein.

10 A "thioaryloxy" group refers to both an -S-aryl and an -S-heteroaryl group, as defined herein.

A "carbonyl" group refers to a $-C(=O)-R$ group, where R" is selected from the group consisting of hydrogen, alkyl, cycloalkyl, aryl, heteroaryl (bonded through a ring carbon) and
15 heteroalicyclic (bonded through a ring carbon), as defined herein.

An "aldehyde" group refers to a carbonyl group where R" is hydrogen.

A "thiocarbonyl" group refers to a $-C(=S)-R$ group, with R"
20 as defined herein.

A "C-carboxy" group refers to a $-C(=O)O-R$ groups, with R" as defined herein.

A "carboxylic acid" group refers to a C-carboxyl group in which R" is hydrogen.

A "halo" group refers to fluorine, chlorine, bromine or iodine.

5 A "trihalomethyl" group refers to a -CX₃ group wherein X is a halo group as defined herein.

A "trihalomethanesulfonyl" group refers to a X₃CS(=O)₂- groups with X as defined above.

10 A "sulfinyl" group refers to a -S(=O)-R" group, with R" as defined herein.

A "sulfonyl" group refers to a -S(=O)₂R" group, with R" as defined herein.

An "S-sulfonamido" group refers to a -S(=O)₂NR¹⁰R¹¹ group, with R¹⁰ and R¹¹ as defined herein.

15 An "N-sulfonamido" group refers to a R¹⁰(=O)₂NR¹¹- group, with R¹⁰ and R¹¹ as defined herein.

A "trihalomethanesulfonamido" group refers to a X₃CS(=O)₂NR¹⁰- group with R¹⁰ as defined herein.

20 An "O-carbamyl" group refers to a -OC(=O)NR¹⁰R¹¹ group with R¹⁰ and R¹¹ as defined herein.

An "N-carbamyl" group refers to a R¹¹OC(=O)NR¹⁰- group, with R¹⁰ and R¹¹ as defined herein.

An "O-thiocarbamyl" group refers to a -OC(=S)NR¹⁰R¹¹ group with R¹⁰ and R¹¹ as defined herein.

An "N-thiocarbamyl" group refers to a $R^{11}OC(=S)NR^{10}$ - group, with R^{10} and R^{11} as defined herein.

An "amino" group refers to an $-NH_2$ group.

A "C-amido" group refers to a $-C(=O)NR^{10}R^{11}$ group with R^{10} and
5 R^{11} as defined herein.

An "N-amido" group refers to a $R^{11}C(=O)NR^{10}$ - group, with R^{10} and R^{11} as defined herein.

A "quaternary ammonium" group refers to a $-NHR^{10}R^{11}$ group wherein R^{10} and R^{11} are independently selected from the group
10 consisting of alkyl, cycloalkyl, aryl, and heteroaryl.

A "ureido" group refers to a $-NR^{10}C(=O)NR^{11}R^{12}$ group, with R^{10} and R^{11} as defined herein and R^{12} defined the same as R^{10} and R^{11} .

A "guanidino" group refers to a $-R^{10}NC(=N)NR^{11}R^{12}$ group, with R^{10} , R^{11} and R^{12} as defined herein.

15 A "guanyl" group refers to a $R^{10}R^{11}NC(=N)$ - group, with R^{10} and R^{11} as defined herein.

A "nitro" group refers to a $-NO_2$ group.

A "cyano" group refers to a $-C\equiv N$ group.

A "silyl" group refers to a $-Si(R)_3$, with R as defined
20 herein.

B. Preferred Structural Features.

Preferred structural features of this invention are those in which:

A and D are nitrogen, B is carbon and either A or D is

participating in a double bond.

The "R" group bonded to whichever of A or D is not participating in a double bond; i.e., R^1 or R^3 , is selected from the group consisting of hydrogen, alkyl, halo, cycloalkyl, aryl, carbonyl and C-carboxy.

R^2 is selected from the group consisting of hydrogen, alkyl, cycloalkyl, trihalomethyl, aryl, hydroxy, alkoxy, aryloxy, O-carboxy, amino and $NR^{10}R^{11}$ where R^{10} and R^{11} are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, aryl, carbonyl, sulfonyl, trihalomethanesulfonyl and, combined, a five- or six-member heteroalicyclic ring.

R^4 and R^7 are independently selected from the group consisting of hydrogen, alkyl, alkoxy, aryloxy, C-carboxy, cycloalkyl, hydroxy and halo.

R^5 and R^6 are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkoxy, aryl, aryloxy, heteroaryl, heteroalicyclic, hydroxy and halo with the proviso that, when one of R^5 or R^6 is hydrogen, methyl or phenyl, the other is not any of hydrogen, methyl or phenyl.

Further preferred embodiments of this invention are those in which:

A is nitrogen which is participating in a double bond.

D is sulfur.

R^2 , R^4 and R^7 are independently selected from the group

consisting of hydrogen, alkyl and cycloalkyl.

R⁵ and R⁶ are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy and aryloxy.

5 Additional preferred embodiments of this invention are those in which:

A is sulfur.

B and D are carbon atoms which are participating in a double bond.

10 R² and R³ are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, aryl, C-carboxy and C-amido.

R⁴ and R⁷ are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, hydroxy, alkoxy,
15 aryloxy and carbonyl.

R⁵ and R⁶ are independently selected from the group consisting of hydrogen, alkyl, aryl, heteroaryl and heteroalicyclic with the proviso that, when one of R⁵ or R⁶ is hydrogen, methyl or phenyl, the other is not any of hydrogen,
20 methyl or phenyl.

The compounds shown in Table 1, infra, comprise still further preferred embodiments of this invention. The substituent designations refer to Formula 1, supra.

TABLE 1

Compound No.	A	B	D	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷
1	N	C	N	CH	CH ₃	-	H	p-CH ₃ Ph	H	H
2	N	C	N	CH ₃	CH ₃	-	H	p-CH ₃ OPh	H	H
3	N	C	N	CH ₃	CH ₃	-	H	p-HOPh	H	H
4	N	C	N	CH ₃	CH ₃	-	H	p-(CH ₃) ₂ NPh	H	H
5	N	C	N	CH ₃	CH ₃	-	H	p-N ₃ Ph	H	H
6	N	C	N	CH ₃	CH ₃	-	H	furan	H	H
7	N	C	N	CH ₃	CH ₃	-	H	H	p-NO ₂ Ph	H
8	N	C	N	CH ₃	CH ₃	-	H	H	p-CH ₃ Ph	H
9	N	C	N	CH ₃	CH ₃	-	H	H	p-CH ₃ OPh	H
10	N	C	N	CH ₃	CH ₃	-	H	H	p-HOPh	H
11	N	C	N	CH ₃	CH ₃	-	H	H	p-(CH ₃) ₂ NPh	H
12	N	C	N	CH ₃	CH ₃	-	H	H	p-N ₃ Ph	H
13	N	C	N	CH ₃	CH ₃	-	H	H	furan	H
14	N	C	N	CH ₃	CH ₃	-	H	p-BrPh	p-BrPh	H
15	N	C	N	CH ₃	CH ₃	-	H	p-CH ₃ Ph	p-CH ₃ Ph	H
16	N	C	N	CH ₃	CH ₃	-	H	p-CH ₃ OPh	p-CH ₃ OPh	H
17	N	C	N	CH ₃	CH ₃	-	H	p-(CH ₃) ₂ NPh	p-(CH ₃) ₂ NPh	H
18	N	C	N	CH ₃	CH ₃	-	H	o-ClPh	o-ClPh	H
19	N	C	N	CH ₃	CH ₃	-	H	m-CH ₃ OPh	m-CH ₃ OPh	H
20	N	C	N	CH ₃	CH ₃	-	H	3-Br-6-OHPh	3-Br-6-OHPh	H
21	N	C	N	CH ₃	CH ₃	-	H	furan-2-yl	furan-2-yl	H
22	N	C	N	CH ₃	CH ₃	-	H	thiophene-2-yl	thiophene-2-yl	H
23	N	C	N	CH ₃	CH ₃	-	H	pyridine-2-yl	pyridine-2-yl	H
24	N	C	N	CH ₃	CH ₃	-	H	6-CH ₃ pyridine-2-yl	6-CH ₃ pyridine-2-yl	H
25	N	C	N	CH ₃	CH ₃	-	CH ₃	Ph	H	H
26	N	C	N	CH ₃	CH ₃	-	CH ₃	H	Ph	H
27	N	C	N	CH ₃	CH ₃	-	CH ₃	Ph	Ph	H
28	N	C	N	CH ₃	CH ₃	-	CH ₃	p-NO ₂ Ph	H	H
29	N	C	N	CH ₃	CH ₃	-	CH ₃	p-CH ₃ Ph	H	H

Compound No.	A	B	D	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷
30	N	C	N	CH ₃	CH ₃	-	CH ₃	p-CH ₃ OPh	H	H
31	N	C	N	CH ₃	CH ₃	-	CH ₃	p-HOPh	H	H
32	N	C	N	CH ₃	CH ₃	-	CH ₃	p-(CH ₃) ₂ NPh	H	H
33	N	C	N	CH ₃	CH ₃	-	CH ₃	p-N ₃ Ph	H	H
34	N	C	N	CH ₃	CH ₃	-	CH ₃	furan	H	H
35	N	C	N	CH ₃	CH ₃	-	CH ₃	H	p-N ₂ Ph	H
36	N	C	N	CH ₃	CH ₃	-	CH ₃	H	p-CH ₃ Ph	H
37	N	C	N	CH ₃	CH ₃	-	CH ₃	H	p-CH ₃ OPh	H
38	N	C	N	CH ₃	CH ₃	-	CH ₃	H	o-OHPH	H
39	N	C	N	CH ₃	CH ₃	-	CH ₃	H	p-(CH ₃) ₂ NPh	H
40	N	C	N	CH ₃	CH ₃	-	CH ₃	H	p-N ₃ Ph	H
41	N	C	N	CH ₃	CH ₃	-	CH ₃	H	furan	H
42	N	C	N	CH ₃	CH ₃	-	CH ₃	p-BrPh	p-BrPh	H
43	N	C	N	CH ₃	CH ₃	-	CH ₃	p-CH ₃ Ph	p-CH ₃ Ph	H
44	N	C	N	CH ₃	CH ₃	-	CH ₃	p-CH ₃ OPh	p-CH ₃ OPh	H
45	N	C	N	CH ₃	CH ₃	-	CH ₃	p-(CH ₃) ₂ NPh	p-(CH ₃) ₂ NPh	H
46	N	C	N	CH ₃	CH ₃	-	CH ₃	o-ClPh	o-ClPh	H
47	N	C	N	CH ₃	CH ₃	-	CH ₃	m-CH ₃ OPh	m-CH ₃ OPh	H
48	N	C	N	CH ₃	CH ₃	-	CH ₃	3-Br-6-OHPH	3-Br-6-OHPH	H
49	N	C	N	CH ₃	CH ₃	-	CH ₃	furan-2-yl	furan-2-yl	H
50	N	C	N	CH ₃	CH ₃	-	CH ₃	thiophene-2-yl	thiophene-2-yl	H
51	N	C	N	CH ₃	CH ₃	-	CH ₃	pyridine-2-yl	pyridine-2-yl	H
52	N	C	N	CH ₃	CH ₃	-	CH ₃	6-CH ₃ pyridine-2-yl	6-CH ₃ pyridine-2-yl	H
53	N	C	N	CH ₃	CH ₃	-	CH ₃	CH ₃	H	H
54	N	C	N	CH ₃	CH ₃	-	CH ₃	H	CH ₃	H
55	N	C	N	CH ₃	CH ₃	-	CH ₃	CH ₃	CH ₃	H
56	N	C	N	CH ₃	CH ₃	-	CH ₃	Ph	H	H
57	N	C	N	CH ₃	CH ₃	-	CH ₃	H	Ph	H
58	N	C	N	CH ₃	CH ₃	-	CH ₃	Ph	Ph	H

Compound No.	A	B	D	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷
59	N	C	N	CH ₃	CH ₃	-	CH ₃	CH ₃	H	H
60	N	C	N	CH ₃	CH ₃	-	CH ₃	H	CH ₃	H
61	N	C	N	CH ₃	CH ₃	-	CH ₃	CH ₃	CH ₃	H
62	N	C	N	CH ₃	CH ₃	-	F	Ph	H	H
63	N	C	N	CH ₃	CH ₃	-	F	H	Ph	H
64	N	C	N	CH ₃	CH ₃	-	F	Ph	Ph	H
65	N	C	N	CH ₃	CH ₃	-	F	p-NO ₂ Ph	H	H
66	N	C	N	CH ₃	CH ₃	-	F	p-CH ₃ Ph	H	H
67	N	C	N	CH ₃	CH ₃	-	F	p-CH ₃ OPh	H	H
68	N	C	N	CH ₃	CH ₃	-	F	p-HOPh	H	H
69	N	C	N	CH ₃	CH ₃	-	F	p-(CH ₃) ₂ NPh	H	H
70	N	C	N	CH ₃	CH ₃	-	F	p-N ₃ Ph	H	H
71	N	C	N	CH ₃	CH ₃	-	F	furan	H	H
72	N	C	N	CH ₃	CH ₃	-	F	H	p-NO ₂ Ph	H
73	N	C	N	CH ₃	CH ₃	-	F	H	p-CH ₃ Ph	H
74	N	C	N	CH ₃	CH ₃	-	F	H	p-CH ₃ OPh	H
75	N	C	N	CH ₃	CH ₃	-	F	H	p-HOPh	H
76	N	C	N	CH ₃	CH ₃	-	F	H	p-(CH ₃) ₂ NPh	H
77	N	C	N	CH ₃	CH ₃	-	F	H	p-N ₃ Ph	H
78	N	C	N	CH ₃	CH ₃	-	F	H	furan	H
79	N	C	N	CH ₃	CH ₃	-	F	p-BrPh	p-BrPh	H
80	N	C	N	CH ₃	CH ₃	-	F	p-CH ₃ Ph	p-CH ₃ Ph	H
81	N	C	N	CH ₃	CH ₃	-	F	p-CH ₃ OPh	p-CH ₃ OPh	H
82	N	C	N	CH ₃	CH ₃	-	F	p-(CH ₃) ₂ NPh	p-(CH ₃) ₂ NPh	H
83	N	C	N	CH ₃	CH ₃	-	F	o-ClPh	o-ClPh	H
84	N	C	N	CH ₃	CH ₃	-	F	m-CH ₃ OPh	m-CH ₃ OPh	H
85	N	C	N	CH ₃	CH ₃	-	F	3-Br-6-OHPh	3-Br-6-OHPh	H
86	N	C	N	CH ₃	CH ₃	-	F	furan-2-yl	furan-2-yl	H
87	N	C	N	CH ₃	CH ₃	-	F	thiophene-2-yl	thiophene-2-yl	H

Compound No.	A	B	D	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷
88	N	C	N	CH ₃	CH ₃	-	F	pyridine-2-yl	pyridine-2-yl	H
89	N	C	N	CH ₃	CH ₃	-	F	6-CH ₃ pyridine-2-yl	6-CH ₃ pyridine-2-yl	H
90	N	C	N	CH ₃	CH ₃	-	COOH	Ph	H	H
91	N	C	N	CH ₃	CH ₃	-	COOH	H	Ph	H
92	N	C	N	CH ₃	CH ₃	-	COOH	Ph	H	H
93	N	C	N	CH ₃	CH ₃	-	COOH	p-NO ₂ Ph	H	H
94	N	C	N	CH ₃	CH ₃	-	COOH	p-CH ₃ Ph	H	H
95	N	C	N	CH ₃	CH ₃	-	COOH	p-CH ₃ OPh	H	H
96	N	C	N	CH ₃	CH ₃	-	COOH	p-HOPh	H	H
97	N	C	N	CH ₃	CH ₃	-	COOH	p-(CH ₃) ₂ NPh	H	H
98	N	C	N	CH ₃	CH ₃	-	COOH	p-N ₃ Ph	H	H
99	N	C	N	CH ₃	CH ₃	-	COOH	furan	H	H
100	N	C	N	CH ₃	CH ₃	-	COOH	H	p-NO ₂ Ph	H
101	N	C	N	CH ₃	CH ₃	-	COOH	H	p-CH ₃ Ph	H
102	N	C	N	CH ₃	CH ₃	-	COOH	H	p-CH ₃ OPh	H
103	N	C	N	CH ₃	CH ₃	-	COOH	H	p-HOPh	H
104	N	C	N	CH ₃	CH ₃	-	COOH	H	p-(CH ₃) ₂ NPh	H
105	N	C	N	CH ₃	CH ₃	-	COOH	H	p-N ₃ Ph	H
106	N	C	N	CH ₃	CH ₃	-	COOH	H	furan	H
107	N	C	N	CH ₃	CH ₃	-	COOH	p-BrPh	p-BrPh	H
108	N	C	N	CH ₃	CH ₃	-	COOH	p-CH ₃ Ph	p-CH ₃ Ph	H
109	N	C	N	CH ₃	CH ₃	-	COOH	p-CH ₃ OPh	p-CH ₃ OPh	H
110	N	C	N	CH ₃	CH ₃	-	COOH	p-(CH ₃) ₂ NPh	p-(CH ₃) ₂ NPh	H
111	N	C	N	CH ₃	CH ₃	-	COOH	o-ClPh	o-ClPh	H
112	N	C	N	CH ₃	CH ₃	-	COOH	m-CH ₃ OPh	m-CH ₃ OPh	H
113	N	C	N	CH ₃	CH ₃	-	COOH	3-Br-6-OHPh	3-Br-6-OHPh	H
114	N	C	N	CH ₃	CH ₃	-	COOH	furan-2-yl	furan-2-yl	H
115	N	C	N	CH ₃	CH ₃	-	COOH	thiophene-2-yl	thiophene-2-yl	H
116	N	C	N	CH ₃	CH ₃	-	COOH	pyridine-2-yl	pyridine-2-yl	H

Compound No.	A	B	D	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷
117	N	C	N	CH ₃	CH ₃	-	COOH	6-CH ₃ pyridine-2-yl	6-CH ₃ pyridine-2-yl	H
118	N	C	N	CH ₃	CH ₃	-	OCH ₃	Ph	H	H
119	N	C	N	CH ₃	CH ₃	-	OCH ₃	H	Ph	H
120	N	C	N	CH ₃	CH ₃	-	OCH ₃	Ph	Ph	H
121	N	C	N	CH ₃	CH ₃	-	OCH ₃	p-NO ₂ Ph	H	H
122	N	C	N	CH ₃	CH ₃	-	OCH ₃	p-CH ₃ Ph	H	H
123	N	C	N	CH ₃	CH ₃	-	OCH ₃	p-CH ₃ OPh	H	H
124	N	C	N	CH ₃	CH ₃	-	OCH ₃	p-HOPh	H	H
125	N	C	N	CH ₃	CH ₃	-	OCH ₃	p-(CH ₃) ₂ NPh	H	H
126	N	C	N	CH ₃	CH ₃	-	OCH ₃	p-N ₃ Ph	H	H
127	N	C	N	CH ₃	CH ₃	-	OCH ₃	furan	H	H
128	N	C	N	CH ₃	CH ₃	-	OCH ₃	H	p-NO ₂ Ph	H
129	N	C	N	CH ₃	CH ₃	-	OCH ₃	H	p-CH ₃ Ph	H
130	N	C	N	CH ₃	CH ₃	-	OCH ₃	H	p-CH ₃ OPh	H
131	N	C	N	CH ₃	CH ₃	-	OCH ₃	H	p-HOPh	H
132	N	C	N	CH ₃	CH ₃	-	OCH ₃	H	p-(CH ₃) ₂ NPh	H
133	N	C	N	CH ₃	CH ₃	-	OCH ₃	H	p-N ₃ Ph	H
134	N	C	N	CH ₃	CH ₃	-	OCH ₃	H	furan	H
135	N	C	N	CH ₃	CH ₃	-	OCH ₃	p-BrPh	p-BrPh	H
136	N	C	N	CH ₃	CH ₃	-	OCH ₃	p-CH ₃ Ph	p-CH ₃ Ph	H
137	N	C	N	CH ₃	CH ₃	-	OCH ₃	p-CH ₃ OPh	p-CH ₃ OPh	H
138	N	C	N	CH ₃	CH ₃	-	OCH ₃	p-(CH ₃) ₂ NPh	p-(CH ₃) ₂ NPh	H
139	N	C	N	CH ₃	CH ₃	-	OCH ₃	o-ClPh	o-ClPh	H
140	N	C	N	CH ₃	CH ₃	-	OCH ₃	m-CH ₃ OPh	m-CH ₃ OPh	H
141	N	C	N	CH ₃	CH ₃	-	OCH ₃	3-Br-6-OHPh	3-Br-6-OHPh	H
142	N	C	N	CH ₃	CH ₃	-	OCH ₃	furan-2-yl	furan-2-yl	H
143	N	C	N	CH ₃	CH ₃	-	OCH ₃	thiophene-2-yl	thiophene-2-yl	H
144	N	C	N	CH ₃	CH ₃	-	OCH ₃	pyridine-2-yl	pyridine-2-yl	H
145	N	C	N	CH ₃	CH ₃	-	OCH ₃	6-CH ₃ pyridine-2-yl	6-CH ₃ pyridine-2-yl	H

Compound No.	A	B	D	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷
146	N	C	N	CH ₃	CH ₃	-	H	p-CH ₃ Ph	H	H
147	N	C	N	CH ₃	CH ₃	-	H	p-CH ₃ OPh	H	H
148	N	C	N	CH ₃	CH ₃	-	H	p-HOPh	H	H
149	N	C	N	CH ₃	CH ₃	-	H	p-(CH ₃) ₂ NPh	H	H
150	N	C	N	CH ₃	CH ₃	-	H	p-N ₃ Ph	H	H
151	N	C	N	CH ₃	CH ₃	-	H	furan	H	H
152	N	C	N	CH ₃	CH ₃	-	H	H	p-NO ₂ Ph	H
153	N	C	N	CH ₃	CH ₃	-	H	H	p-CH ₃ Ph	H
154	N	C	N	CH ₃	CH ₃	-	H	H	p-CH ₃ OPh	H
155	N	C	N	CH ₃	CH ₃	-	H	H	p-HOPh	H
156	N	C	N	CH ₃	CH ₃	-	H	H	p-(CH ₃) ₂ NPh	H
157	N	C	N	CH ₃	CH ₃	-	H	H	p-N ₃ Ph	H
158	N	C	N	CH ₃	CH ₃	-	H	H	furan	H
159	N	C	N	CH ₃	CH ₃	-	H	p-BrPh	p-BrPh	H
160	N	C	N	CH ₃	CH ₃	-	H	p-CH ₃ Ph	p-CH ₃ Ph	H
161	N	C	N	CH ₃	CH ₃	-	H	p-CH ₃ OPh	p-CH ₃ OPh	H
162	N	C	N	CH ₃	CH ₃	-	H	p-(CH ₃) ₂ NPh	p-(CH ₃) ₂ NPh	H
163	N	C	N	CH ₃	CH ₃	-	H	o-ClPh	o-ClPh	H
164	N	C	N	CH ₃	CH ₃	-	H	m-CH ₃ OPh	m-CH ₃ OPh	H
165	N	C	N	CH ₃	CH ₃	-	H	3-Br-6-OHPh	3-Br-6-OHPh	H
166	N	C	N	CH ₃	CH ₃	-	H	furan-2-yl	furan-2-yl	H
167	N	C	N	CH ₃	CH ₃	-	H	thiophene-2-yl	thiophene-2-yl	H
168	N	C	N	CH ₃	CH ₃	-	H	pyridine-2-yl	pyridine-2-yl	H
169	N	C	N	CH ₃	CH ₃	-	H	6-CH ₃ pyridine-2-yl	6-CH ₃ pyridine-2-yl	H
170	N	C	N	CH ₃	CH ₃	-	H	Ph	H	H
171	N	C	N	CH ₃	CH ₃	-	H	H	Ph	H
172	N	C	N	CH ₃	CH ₃	-	H	Ph	Ph	H
173	N	C	N	CH ₃	CH ₃	-	H	CH ₃	H	H
174	N	C	N	CH ₃	CH ₃	-	H	H	CH ₃	H

Compound No.	A	B	D	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷
175	N	C	N	CH ₃	CH ₃	-	H	CH ₃	CH ₃	H
176	N	C	N	CH ₃	CH ₃	-	CH ₃	p-CH ₃ Ph	H	H
177	N	C	N	CH ₃	CH ₃	-	CH ₃	p-CH ₃ OPh	H	H
178	N	C	N	CH ₃	CH ₃	-	CH ₃	p-HOPh	H	H
179	N	C	N	CH ₃	CH ₃	-	CH ₃	p-(CH ₃) ₂ NPh	H	H
180	N	C	N	CH ₃	CH ₃	-	CH ₃	p-N ₃ Ph	H	H
181	N	C	N	CH ₃	CH ₃	-	CH ₃	furan	H	H
182	N	C	N	CH ₃	CH ₃	-	CH ₃	H	p-NO ₂ Ph	H
183	N	C	N	CH ₃	CH ₃	-	CH ₃	H	p-CH ₃ Ph	H
184	N	C	N	CH ₃	CH ₃	-	CH ₃	H	p-CH ₃ OPh	H
185	N	C	N	CH ₃	CH ₃	-	CH ₃	H	p-HOPh	H
186	N	C	N	CH ₃	CH ₃	-	CH ₃	H	p-(CH ₃) ₂ NPh	H
187	N	C	N	CH ₃	CH ₃	-	CH ₃	H	p-N ₃ Ph	H
188	N	C	N	CH ₃	CH ₃	-	CH ₃	H	furan	H
189	N	C	N	CH ₃	CH ₃	-	CH ₃	p-BrPh	p-BrPh	H
190	N	C	N	CH ₃	CH ₃	-	CH ₃	p-CH ₃ Ph	p-CH ₃ Ph	H
191	N	C	N	CH ₃	CH ₃	-	CH ₃	p-CH ₃ OPh	p-CH ₃ OPh	H
192	N	C	N	CH ₃	CH ₃	-	CH ₃	p-(CH ₃) ₂ NPh	p-(CH ₃) ₂ NPh	H
193	N	C	N	CH ₃	CH ₃	-	CH ₃	o-ClPh	o-ClPh	H
194	N	C	N	CH ₃	CH ₃	-	CH ₃	m-CH ₃ OPh	m-CH ₃ OPh	H
195	N	C	N	CH ₃	CH ₃	-	CH ₃	3-Br-6-OHPh	3-Br-6-OHPh	H
196	N	C	N	CH ₃	CH ₃	-	CH ₃	furan-2-yl	furan-2-yl	H
197	N	C	N	CH ₃	CH ₃	-	CH ₃	thiophene-2-yl	thiophene-2-yl	H
198	N	C	N	CH ₃	CH ₃	-	CH ₃	pyridine-2-yl	pyridine-2-yl	H
199	N	C	N	CH ₃	CH ₃	-	CH ₃	6-CH ₃ pyridine-2-yl	6-CH ₃ pyridine-2-yl	H
200	N	C	N	CH ₃	CH ₃	-	CH ₃	Ph	H	H
201	N	C	N	CH ₃	CH ₃	-	CH ₃	H	Ph	H
202	N	C	N	CH ₃	CH ₃	-	CH ₃	Ph	Ph	H
203	N	C	N	CH ₃	CH ₃	-	CH ₃	CH ₃	CH ₃	H

Compound No.	A	B	D	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷
204	N	C	N	CH ₃	CH ₃	-	H	H	p-NO ₂ Ph	H
205	N	C	N	CH ₃	CH ₃	-	H	H	p-CH ₃ Ph	H
206	N	C	N	CH ₃	CH ₃	-	H	H	p-CH ₃ OPh	H
207	N	C	N	CH ₃	CH ₃	-	H	H	p-HOPh	H
208	N	C	N	CH ₃	CH ₃	-	H	H	p-(CH ₃) ₂ NPh	H
209	N	C	N	CH ₃	CH ₃	-	H	H	p-N ₃ Ph	H
210	N	C	N	CH ₃	CH ₃	-	H	H	furan	H
211	N	C	N	CH ₃	CH ₃	-	H	p-BrPh	p-BrPh	H
212	N	C	N	CH ₃	CH ₃	-	H	p-CH ₃ Ph	p-CH ₃ Ph	H
213	N	C	N	CH ₃	CH ₃	-	H	p-CH ₃ OPh	p-CH ₃ OPh	H
214	N	C	N	CH ₃	CH ₃	-	H	p-(CH ₃) ₂ NPh	p-(CH ₃) ₂ NPh	H
215	N	C	N	CH ₃	CH ₃	-	H	o-ClPh	o-ClPh	H
216	N	C	N	CH ₃	CH ₃	-	H	m-CH ₃ OPh	m-CH ₃ OPh	H
217	N	C	N	CH ₃	CH ₃	-	H	3-Br-6-OHPh	3-Br-6-OHPh	H
218	N	C	N	CH ₃	CH ₃	-	H	furan-2-yl	furan-2-yl	H
219	N	C	N	CH ₃	CH ₃	-	H	thiophene-2-yl	thiophene-2-yl	H
220	N	C	N	CH ₃	CH ₃	-	H	pyridine-2-yl	pyridine-2-yl	H
221	N	C	N	CH ₃	CH ₃	-	H	6-CH ₃ pyridine-2-yl	6-CH ₃ pyridine-2-yl	H
222	N	C	N	CH ₃	CH ₃	-	H	Ph	H	H
223	N	C	N	CH ₃	CH ₃	-	H	H	Ph	H
224	N	C	N	CH ₃	CH ₃	-	H	Ph	Ph	H
225	N	C	N	CH ₃	CH ₃	-	H	CH ₃	H	H
226	N	C	N	CH ₃	CH ₃	-	H	H	CH ₃	H
227	N	C	N	CH ₃	CH ₃	-	H	CH ₃	CH ₃	H

2. THE BIOCHEMISTRY

In yet another embodiment, this invention relates to a method for the modulation of the catalytic activity of PTKs comprising administering a compound of this invention or a physiologically acceptable salt or a prodrug thereof to a PTK.

By "PTK" is meant both RTKs and CTKs; i.e., the modulation of both RTK signal transduction and CTK signal transduction is contemplated by this invention.

The term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "modulation" or "modulating" refers to the alteration of the catalytic activity of RTKs and/or CTKs. In particular, modulating refers to the activation of the catalytic activity of RTKs and/or CTKs, more preferably to the activation or inhibition of the catalytic activity of RTKs and/or CTKs, depending on the concentration of the compound administered or, more preferably still, to the inhibition of the catalytic activity of RTKs and/or CTKs.

The term "catalytic activity" as used herein refers to the

rate of phosphorylation of tyrosine under the influence, direct or indirect, of RTKs and/or CTKs.

The term "administering" as used herein refers to a method for bringing a compound of this invention and a target PK together in such a manner that the compound can affect the catalytic activity of the PK either directly; i.e., by interacting with the kinase itself, or indirectly; i.e., by interacting with another molecule on which the catalytic activity of the kinase is dependent. As used herein, administration can be accomplished either in vitro, i.e. in a test tube, or in vivo, i.e. in cells or tissues of a living organism. Thus, the PTK mediated disorders which are the object of this invention can be studied, prevented or treated by the methods set forth herein whether the cells or tissues of the organism exist within the organism or outside the organism. Cells existing outside the organism can be maintained or grown in cell culture dishes. In this context, the ability of a particular compound to affect a PTK related disorder can be determined; i.e., the IC50 of the compound, defined below, can be ascertained before the use of the compounds in more complex living organisms is attempted. For cells outside the organism, multiple methods exist, and are well-known to those skilled in the art, to administer compounds including, but not limited to, direct cell microinjection and numerous transmembrane carrier techniques. For cells harbored

within a living organism, myriad methods also exist, and are likewise well-known to those skilled in the art, to administer compounds including, but not limited to, oral, parenteral, dermal and aerosol applications.

5 RTK mediated signal transduction is initiated by extracellular interaction with a specific growth factor (ligand), followed by receptor dimerization, transient stimulation of the intrinsic protein tyrosine kinase activity and phosphorylation. Binding sites are thereby created for intracellular signal
10 transduction molecules and lead to the formation of complexes with a spectrum of cytoplasmic signaling molecules that facilitate the appropriate cellular response (e.g., cell division or metabolic responses to the extracellular microenvironment). See, Schlessinger and Ullrich, 1992, Neuron 9:303-391.

15 It has been shown that tyrosine phosphorylation sites in growth factor receptors function as high-affinity binding sites for SH2 (src homology) domains of signaling molecules. Fantl et al., 1992, Cell 69:413-423; Songyang et al., 1994, Mol. Cell. Biol. 14:2777-2785; Songyang et al., 1993, Cell 72:767-778; and
20 Koch et al., 1991, Science 252:668-678. Several intracellular substrate proteins that associate with RTKs have been identified. They may be divided into two principal groups: (1) substrates which have a catalytic domain; and (2) substrates which lack such domain but serve as adapters and associate with catalytically

active molecules. Songyang et al., 1993, Cell 72:767-778. The specificity of the interactions between receptors and SH2 domains of their substrates is determined by the amino acid residues immediately surrounding the phosphorylated tyrosine residue.

5 Differences in the binding affinities between SH2 domains and the amino acid sequences surrounding the phosphotyrosine residues on particular receptors are consistent with the observed differences in their substrate phosphorylation profiles. Songyang et al., 1993, Cell 72:767-778. These observations suggest that the
10 function of each RTK is determined not only by its pattern of expression and ligand availability but also by the array of downstream signal transduction pathways that are activated by a particular receptor. Thus, phosphorylation provides an important regulatory step which determines the selectivity of signaling
15 pathways recruited by specific growth factor receptors, as well as differentiation factor receptors.

PTK signal transduction results in, among other responses, cell proliferation, differentiation, growth and metabolism. Abnormal cell proliferation may result in a wide array of
20 disorders and diseases, including the development of neoplasia such as carcinoma, sarcoma, leukemia, glioblastoma and hemangioma; psoriasis, arteriosclerosis, arthritis and diabetic retinopathy and other disorders related to uncontrolled angiogenesis and/or vasculogenesis.

A precise understanding of the mechanism by which the compounds of this invention inhibit PTKs is not required in order to practice the present invention. However, while not being bound to any particular mechanism or theory, it is believed that the compounds interact with the amino acids of the catalytic region of PTKs. PTKs typically possess a bi-lobate structure wherein ATP appears to bind in the cleft between the two lobes in a region where the amino acids are conserved among PTKs. Inhibitors of PTKs are believed to bind by non-covalent interactions such as hydrogen bonding, van der Waals forces and ionic interactions in the same general region where the aforesaid ATP binds to the PTKs. More specifically, it is thought that the quinoxaline ring component of the compounds of this invention binds in the general space normally occupied by the adenine ring of ATP. Specificity of a particular quinoxaline for a particular PTK may arise as the result of additional interactions between the various substituents on the quinoxaline core and the amino acid domain specific to particular PTKs. That is, different quinoxaline substituents may contribute to preferential binding to particular PTKs. The ability to select compounds active at different ATP (or other nucleotide) binding sites makes the compounds of this invention useful for targeting any protein with such a site; i.e., not only PTKs but serine/threonine kinases and protein phosphatases as well. Thus, the compounds disclosed

herein may be useful as in vitro assays for such proteins as well as in vivo therapeutic agents acting through such proteins.

Thus, in another aspect, this invention relates to a method for treating or preventing a PTK related disorder by

5 administering a therapeutically effective amount of a compound of this invention or a salt or a prodrug thereof to an organism.

In addition to the compounds described above, the compounds shown in Table 2, below, are expected to be useful in treating or preventing PTK related disorders in an organism.

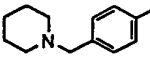
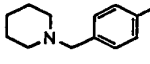
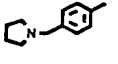
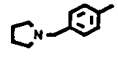
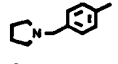

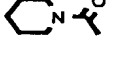
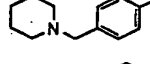
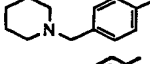
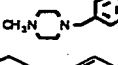
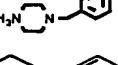
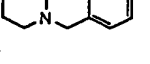
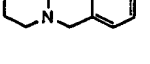
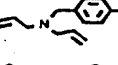
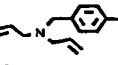
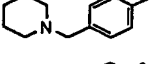
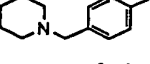
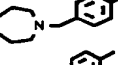
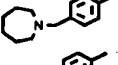
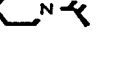
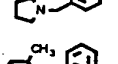
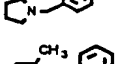
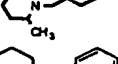
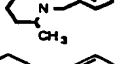
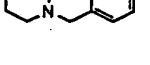
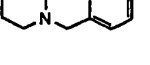
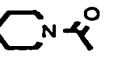
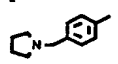
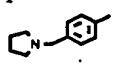
10 As used herein, "PTK related disorder," "PTK driven disorder," and "abnormal PTK activity" all refer to a disorder characterized by inappropriate PTK activity or over-activity of the PTK, which can be either an RTK or a CTK. Inappropriate activity refers to either: (1) PTK expression in cells which
15 normally do not express PTKs; (2) increased PTK expression leading to unwanted cell proliferation, differentiation and/or growth; or, (3) decreased PTK expression leading to unwanted reductions in cell proliferation, differentiation and/or growth.

Overactivity of PTKs refers to either amplification of the gene
20 encoding a particular PTK or production of a level of PTK activity which can correlate with a cell proliferation, differentiation and/or growth disorder (that is, as the level of the PTK increases, the severity of one or more of the symptoms of the cellular disorder increases).

As used herein, the terms "prevent", "preventing" and "prevention" refer to a method for barring an organism from acquiring a PTK mediated cellular disorder in the first place.

TABLE 2

Compound										
No.	A	B	D	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷
228	N	C	N	H	OH	-	H	Ph	Ph	H
229	N	C	N	Cl	CF ₃	-	H	Cl	Cl	H
230	N	C	N	H	CH ₃	-	H	Ph	Ph	H
231	N	C	N	CH ₃	CH ₃	-	H	Ph	Ph	H
232	N	C	N	CH ₃ C(=O)-	CH ₃	-	H	CH ₃	CH ₃	H
233	N	C	N	CH ₃	CH ₃	-	H	H	H	H
234	N	C	N	CH ₃	CH ₃	-	H	CH ₃	CH ₃	H
235	N	C	N	CH ₃	NH ₂	-	H	H	CH ₃	CH ₃
236	N	C	N	CH ₃	CH ₃	-	H	Ph	H	H
237	N	C	N	CH ₃	CH ₃	-	H	H	Ph	H
238	N	C	N	H	CH ₃	-	H	H	Ph	H
239	N	C	N	CH ₃	CH ₃	-	H	H	CH ₃	CH ₃
240	O	N	C	-	p-CH ₃ Ph-	-OCH ₃	p-CH ₃ Ph-	p-CH ₃ Ph-	-OCH ₃	H
241	N	N	C	CH ₃	H	-OCH ₃	p-CH ₃ Ph-	p-CH ₃ Ph-	-OCH ₃	H
242	N	C	C	H	H	H	H	Ph	Ph	H
243	S	C	N	-	CH ₃	-	H	OH	CH ₃	H
244	S	C	N	-	CH ₃	-	H	OH	OH	H
245	S	C	N	-	H	-	H	CH ₃	CH ₃	H
246	S	C	N	-	CH ₃	-	H	Ph	H	H
247	S	C	N	-	CH ₃	-	H	H	Ph	H
248	S	C	N	-	CH ₃	-	H	CH ₃	CH ₃	H
249	S	C	N	-	CH ₃	-	H	Ph	Ph	H
250	S	C	N	-	CH ₃	-	H	H	H	H

Compound										
No.	A	B	D	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷
251	S	C	N	-	CH ₃	-	H	CH ₃	OH	H
252	S	C	N	-	CH ₃ OC(=O)-	CH ₃	-OCH ₃	Et ₂ NCH ₂ Ph-	Et ₂ NCH ₂ Ph-	H
253	S	C	N	-	CH ₃ OC(=O)-	CH ₃	-OCH ₃	Me ₂ NCH ₂ Ph-	Me ₂ NCH ₂ Ph-	H
254	S	C	N	-	H ₂ NC(=O)-	CH ₃	-OCH ₃			H
255	S	C	N	-	CH ₃ OC(=O)-	CH ₃	-OCH ₃			H
256	S	C	N	-	HOC(-O)-	CH ₃	-OCH ₃			H
257	S	C	N	-		H ₃	-OCH ₃			H
258	S	C	N	-	CH ₃ OC(=O)-	H ₃	-OCH ₃			H
259	S	C	N	-	Et ₂ NC(=O)-	CH ₃	-OCH ₃			H
260	S	C	N	-	CH ₃ OC(=O)-	CH ₃	-OCH ₃	nPr ₂ NCH ₂ Ph-	nPr ₂ NCH ₂ Ph-	H
261	S	C	N	-	CH ₃ O(C=O)-	CH ₃	-OCH ₃			H
262	S	C	N	-	CH ₃ O(C=O)-	CH ₃	-OCH ₃			H
263	S	C	N	-	CH ₃ O(C=O)-	CH ₃	-OCH ₃			H
264	S	C	N	-		CH ₃	-OCH ₃			H
265	S	C	N	-	CH ₃ O(C=O)-	CH ₃	-OCH ₃			H
266	S	C	N	-	HO(C=O)-	CH ₃	-OCH ₃			H
267	S	C	N	-	CH ₃ OC(=O)-	CH ₃	-OCH ₃	p-CH ₃ Ph-	p-CH ₃ Ph-	H
268	S	C	N	-		CH ₃	-OCH ₃			H
269	S	C	N	-	CH ₃ O(C=O)-	CH ₃	-OCH ₃	p-BrCH ₂ Ph-	p-BrCH ₂ Ph-	H
270	N	C	C	CH ₃	H	H	H	H	H	H

As used herein, the terms "treat", "treating" and "treatment" refer to a method of alleviating or abrogating the PTK mediated cellular disorder and/or its attendant symptoms. With regard particularly to cancer, these terms simply mean that the life expectancy of an individual affected with a cancer will be increased or that one or more of the symptoms of the disease will be reduced.

As used herein, the term "cancer" refers to various types of malignant neoplasms, most of which can invade surrounding tissues, and may metastasize to different sites, as defined by Stedman's Medical Dictionary 25th edition (Hensyl ed. 1990). Examples of cancers which may be treated by the present invention include, but are not limited to, brain, ovarian, colon, prostate, kidney, bladder, breast, lung, oral and skin cancers which exhibit inappropriate PTK activity. These cancers can be further broken down. For example, brain cancers include glioblastoma multiforme, anaplastic astrocytoma, astrocytoma, ependymoma, oligodendroglioma, medulloblastoma, meningioma, sarcoma, hemangioblastoma, and pineal parenchymal. Likewise, skin cancers include melanoma and Kaposi's sarcoma.

The term "organism" refers to any living entity comprised of at least one cell. A living organism can be as simple as, for example, a single eukariotic cell or as complex as a mammal, including a human being.

The term "therapeutically effective amount" as used herein refers to that amount of the compound being administered which will relieve to some extent one or more of the symptoms of the disorder being treated. In reference to the treatment of cancer, a therapeutically effective amount refers to that amount which has the effect of (1) reducing the size of the tumor; (2) inhibiting (that is, slowing to some extent, preferably stopping) tumor metastasis; (3) inhibiting to some extent (that is, slowing to some extent, preferably stopping) tumor growth; and/or, (4) relieving to some extent (or preferably eliminating) one or more symptoms associated with the cancer.

This invention is therefore directed to compounds which modulate PTK signal transduction by affecting the enzymatic activity of the RTKs and/or CTKs and thereby interfering with the signal transduced by such proteins. More particularly, the present invention is directed to compounds which modulate the RTK and/or CTK mediated signal transduction pathways as a therapeutic approach to cure many kinds of solid tumors, including but not limited to carcinoma, sarcoma, erythroblastoma, glioblastoma, meningioma, astrocytoma, melanoma and myoblastoma. Indications may include, but are not limited to, brain cancers, bladder cancers, ovarian cancers, gastric cancers, pancreas cancers, colon cancers, blood cancers, lung cancers, bone cancers and leukemias.

Further examples, without limitation, of the types of disorders related to unregulated PTK activity that the compounds described herein may be useful in preventing, treating and studying are cell proliferative disorders, fibrotic disorders and metabolic disorders.

5 Cell proliferative disorders which may be prevented, treated or further studied by the present invention include cancer, blood vessel proliferative disorders and mesangial cell proliferative disorders.

10 Blood vessel proliferative disorders refer to angiogenic and vasculogenic disorders generally resulting in abnormal proliferation of blood vessels. The formation and spreading of blood vessels, or vasculogenesis and angiogenesis, respectively, play important roles in a variety of physiological processes such as embryonic development, corpus luteum formation, wound healing
15 and organ regeneration. They also play a pivotal role in cancer development. Other examples of blood vessel proliferation disorders include arthritis, where new capillary blood vessels invade the joint and destroy cartilage, and ocular diseases, like diabetic retinopathy, where new capillaries in the retina invade
20 the vitreous, bleed and cause blindness. Conversely, disorders related to the shrinkage, contraction or closing of blood vessels, such as restenosis, are also implicated.

Fibrotic disorders refer to the abnormal formation of extracellular matrices. Examples of fibrotic disorders include

hepatic cirrhosis and mesangial cell proliferative disorders.

Hepatic cirrhosis is characterized by the increase in extracellular matrix constituents resulting in the formation of a hepatic scar. Hepatic cirrhosis can lead to diseases such as cirrhosis of the liver. An increased extracellular matrix resulting in a hepatic scar can also be caused by viral infection such as hepatitis. Lipocytes appear to play a major role in hepatic cirrhosis. Other fibrotic disorders implicated include atherosclerosis.

Mesangial cell proliferative disorders refer to disorders brought about by abnormal proliferation of mesangial cells. Mesangial proliferative disorders include various human renal diseases, such as glomerulonephritis, diabetic nephropathy, malignant nephrosclerosis, thrombotic microangiopathy syndromes, transplant rejection, and glomerulopathies. In this regard, PDGFR has been implicated in the maintenance of mesangial cell proliferation. Floege et al., 1993, Kidney International 43:47S-54S.

As noted previously, PTKs have been associated with the development of cancer. Some of these receptors, like EGFR (Tuzi et al., 1991, Br. J. Cancer 63:227-233; Torp et al., 1992, APMIS 100:713-719); HER2/neu (Slamon et al., 1989, Science 244:707-712) and PDGFR (Kumabe et al., 1992, Oncogene, 7:627-633) are over-expressed in many tumors and/or are persistently activated by

autocrine loops. In fact, in the most common and severe cancers, such receptor over-expression and autocrine loops have been demonstrated (Akbasak and Suner-Akbasak et al., 1992, J. Neurol. Sci., 111:119-133; Dickson et al., 1992, Cancer Treatment Res. 61:249-273; Korc et al., 1992, J. Clin. Invest. 90:1352-1360); (Lee and Donoghue, 1992, J. Cell. Biol., 118:1057-1070; Korc et al., supra; Akbasak and Suner-Akbasak et al., supra). For example, the EGFR receptor has been associated with squamous cell carcinoma, astrocytoma, glioblastoma, head and neck cancer, lung cancer and bladder cancer. HER2 has been associated with breast, ovarian, gastric, lung, pancreas and bladder cancer. PDGFR has been associated with glioblastoma, melanoma and lung, ovarian, and prostate cancer. The RTK c-met has been generally associated with hepatocarcinogenesis and thus hepatocellular carcinoma. Additionally, c-met has been linked to malignant tumor formation. More specifically, c-met has been associated with, among other cancers, colorectal, thyroid, pancreatic and gastric carcinoma, leukemia and lymphoma. Additionally, over-expression of the c-met gene has been detected in patients with Hodgkins disease and Burkitts disease.

IGF-IR, in addition to being implicated in nutritional support and in type-II diabetes, has also been associated with several types of cancers. For example, IGF-I has been implicated as an autocrine growth stimulator for several tumor types

including human breast cancer carcinoma cells (Arteaga et al., 1989, J. Clin. Invest. 84:1418-1423) and small lung tumor cells (Macauley et al., 1990, Cancer Res., 50:2511-2517). In addition, IGF-I, while being integrally involved in the normal growth and differentiation of the nervous system, appears to be an autocrine stimulator of human gliomas. Sandberg-Nordqvist et al., 1993, Cancer Res. 53:2475-2478. The importance of the IGF-IR and its ligands in cell proliferation is further supported by the fact that many cell types in culture (fibroblasts, epithelial cells, smooth muscle cells, T-lymphocytes, myeloid cells, chondrocytes and osteoblasts (the stem cells of the bone marrow)) are stimulated to grow by IGF-I. Goldring and Goldring, 1991, Eukaryotic Gene Expression, 1:301-326. In a series of recent publications, Baserga even suggests that IGF-IR plays a central role in the mechanisms of transformation and, as such, could be a preferred target for therapeutic interventions for a broad spectrum of human malignancies. Baserga, 1995, Cancer Res., 55:249-252; Baserga, 1994, Cell 79:927-930; Coppola et al., 1994, Mol. Cell. Biol., 14:4588-4595.

The association between abnormal RTK activity and disease are not restricted to cancer. For example, RTKs have been associated with metabolic diseases like psoriasis, diabetes mellitus, wound healing, inflammation, and neurodegenerative diseases. For example, EGFR has been indicated in corneal and

dermal wound healing. Defects in the Insulin-R and IGF-1R are indicated in type-II diabetes mellitus. A more complete correlation between specific RTKs and their therapeutic indications is set forth in Plowman et al., 1994, DN&P 7:334-339.

5 As noted previously, not only RTKs but CTKs as well including, but not limited to, src, abl, fps, yes, fyn, lyn, lck, blk, hck, fgr and yrk (reviewed by Bolen et al., 1992, FASEB J., 6:3403-3409) are involved in the proliferative and metabolic signal transduction pathway and thus were expected, and have been
10 shown, to be involved in many PTK-mediated disorders to which the present invention is directed. For example, mutated src (v-src) has been demonstrated as an oncoprotein (pp60^{v-src}) in chicken. Moreover, its cellular homolog, the proto-oncogene pp60^{c-src} transmits oncogenic signals of many receptors. Over-expression
15 of EGFR or HER2/neu in tumors leads to the constitutive activation of pp60^{c-src}, which is characteristic for the malignant cell but absent from the normal cell. On the other hand, mice deficient in the expression of c-src exhibit an osteopetrotic phenotype, indicating a key participation of c-src in osteoclast
20 function and a possible involvement in related disorders.

Similarly, Zap70 is implicated in T-cell signaling.

Finally, both RTKs and CTKs are currently suspected as being involved in hyperimmune disorders.

3. PHARMACOLOGICAL COMPOSITIONS AND THERAPEUTIC APPLICATIONS

A compound of the present invention, or its physiologically acceptable salt or prodrug, can be administered to a human patient per se, or in pharmacological compositions where it is mixed with suitable carriers or excipient(s). Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

A. Routes Of Administration.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into a solid tumor, often in a depot or sustained release formulation.

Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with a tumor-specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

B. Composition/Formulation.

Another aspect of this invention relates to pharmaceutical compositions of the compounds described herein and the physiologically acceptable salts and prodrugs thereof.

5 Pharmacological compositions of the present invention may be manufactured by processes well known in the art; e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

10 Pharmacological compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharma-
15 ceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the compounds of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or
20 physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically

acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient.

5 Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, 10 including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose; and/or physiologically acceptable 15 polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this 20 purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee

coatings for identification or to characterize different combinations of active compound doses.

Pharmacological compositions which can be used orally include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and

cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds described herein may be formulated for
5 parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers with, optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles,
10 and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmacological compositions for parenteral administration include aqueous solutions of the active compounds in water soluble form. Additionally, suspensions of the active compounds
15 may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity
20 of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

The compounds of this invention may also be formulated in
5 rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, a compound of this invention may also be formulated as a depot
10 preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or
15 ion exchange resins, or as sparingly soluble derivatives such as sparingly soluble salts.

The pharmacological compositions herein also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include, but are not limited to,
20 calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin and polymers such as polyethylene glycols.

Many of the PTK modulating compounds of the invention may be provided as physiologically acceptable salts wherein the claimed

compound may form the negatively or the positively charged species. Examples of salts in which the compound forms the positively charged moiety include, without limitation, quaternary ammonium (defined elsewhere herein), salts such as the
5 hydrochloride, sulfate, carbonate, lactate, tartrate, maleate, succinate, etc. wherein the nitrogen of the quaternary ammonium group is a nitrogen of a compound of this invention which reacts with an appropriate acid. Salts in which the compound forms the negatively charged species include, without limitation, the
10 sodium, potassium, calcium and magnesium salts formed by the reaction of a carboxylic acid group in the molecule with the appropriate base (e.g. sodium hydroxide (NaOH), potassium hydroxide (KOH), Calcium hydroxide (Ca(OH)₂), etc.).

C. Dosage.

15 Pharmacological compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose.

More specifically, a therapeutically effective amount means an amount of compound effective to prevent, alleviate or
20 ameliorate symptoms of disease or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any compound used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal inhibition of the PTK activity). Such information can be used to more accurately determine useful doses in humans.

10 Toxicity and therapeutic efficacy of the compounds described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., by determining the IC_{50} and the LD_{50} (both of which are discussed further, infra) for a subject compound. The data obtained from these cell culture
15 assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's
20 condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the kinase modulating effects, termed the minimal

effective concentration (MEC). The MEC will vary for each compound but can be estimated from in vitro data; e.g., the concentration necessary to achieve 50-90% inhibition of a kinase may be ascertained using the assays described herein. Dosages
5 necessary to achieve the MEC will depend on individual characteristics and route of administration. HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using the MEC value. Compounds should be administered using a regimen which maintains
10 plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

It is noted that, in the case of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration. In such cases, other
15 procedures known in the art can be employed to determine the effective local concentration.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of
20 the prescribing physician, etc.

D. Packaging.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing

the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

The pack or dispenser may also be accompanied by a notice

5 associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of the labeling
10 approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an
15 indicated condition. Suitable conditions indicated on the label may include treatment of a tumor, inhibition of angiogenesis, treatment of fibrosis, diabetes, and the like.

4. BRIEF DESCRIPTION OF THE TABLES.

20 Table 1 shows the chemical structure of specific compounds of this invention. The compounds shown are not to be construed as limiting the scope of this invention in any manner whatsoever.

Table 2 shows the chemical structures of compounds which are not claimed herein as composition of matter but, rather, are

claimed for their expected utility as modulators of PTK activity and for the treatment and prevention of PTK-related disorders.

Table 3 shows the results of biological assays of some compounds of this invention. These results are provided as
5 examples only and are not to be construed as limiting the scope of this invention in any manner including compound structure, the PTK against which the demonstrative compounds show activity or the level of activity (IC50) shown. PDGFR, FLK-1R and EGFR Kinase are defined and discussed elsewhere herein. IC50 refers
10 to that amount of the tested compound needed to effect a 50% change in the activity of the PTK in comparison with a control in which no compound of this invention is present. With regard to the tests in the table, the 50% change being evaluated is a 50% inhibition of PTK activity compared to that in a control.

15

5. SYNTHESIS

The compounds of this invention may be readily synthesized using techniques well known in the chemical arts. Other synthetic pathways for forming the compounds of the invention
20 will be apparent to those skilled in the art and are deemed to be within the scope and spirit of this invention.

A. Examples.

The following are examples of the synthesis of specific tricyclic quinoxaline compounds of this invention. These

syntheses are provided by way of example only are not to be construed as limiting in any manner.

Example 1. 1,2-Dimethyl-6,7-bis(4-bromo-phenyl)imidazo[4,5-glquinoxaline (ref: J. Gen. Chem. USSR (Engl. Transl.), EN, 1962, 32, 2829-2838).

Step 1: 2-methyl-5,6-dinitrobenzimidazole: To a solution of 2-methyl-5-nitrobenzimidazole in concentrated sulfuric acid was added fuming nitric acid at -5°C. Upon addition of the fuming nitric acid, the reaction mixture was warmed to 0°C held for 1h and poured into crushed ice. The precipitate which formed was filtered, washed with water, and dried overnight at 40°C to give the title compound.

Step 2: 1,2-Dimethyl-5,6-dinitrobenzimidazole: To a solution of 2-methyl-5,6-dinitro-benzimidazole in 5% NaOH was added methanol and dimethyl sulfate. The reaction mixture was heated for 30 min at 90-95°C and then cooled to room temperature. The precipitate which formed was filtered, washed with 10% NaOH and then water, and dried overnight at 40°C to yield 1,2-dimethyl-5,6-dinitrobenzimidazole.

Step 3: 1,2-Dimethyl-5,6-diaminobenzimidazole: 1,2-Dimethyl-5,6- dinitrobenzimidazole was reduced with tin in hydrochloric acid. The solution obtained from the reduction was diluted with water, cooled to 10°C, and treated with 40% NaOH.

The precipitate which formed was filtered, washed with water and recrystallized from alcohol to yield 1,2 dimethly-5,6 diaminobenzimidazole.

Step 4: 1,2-Dimethyl-6,7-bis(4-bromophenyl)imidazo[4,5-

5 g]quinoxaline: To a solution of 1,2-dimethyl-5,6-diaminobenzoimidazole in methanol was added 4,4'-dibromo- benzil. The reaction mixture was refluxed for 3h and cooled to room temperature. The precipitate which formed was filtered and recrystallized from n-butyl alcohol to give the title quinoxaline.

10 Example 2. 1,2,9-Trimethyl-6,7-bis(4-bromophenyl)-imidazol[4,5-g]quinoxaline (ref: Chem. Pharm. Bull. 1994, 42(2), 408-409.

3-Fluoro-2-methylaniline was acetylated with acetic acid anhydride to give 3-fluoro-2-methyl-N-acetylaniline. Displacing
15 fluorine of the 3-fluoro-2-methyl-N-acetylaniline with methylamine followed by nitration, deacetylation and reduction of the nitro group with palladium or charcoal gave 1,2-diamino-3-methyl-4-methylaminobenzene. Condensation of 4,4'-dibromo-
benzil with the 1,2-diamino-3-methyl-4-methylaminobenzene in
20 refluxing methanol gave 2,3-bis(4-bromophenyl)-5-methyl-6-methylaminoquinoxaline which upon acetylation with acetic acid anhydride, nitration, reduction with palladium on charcoal and cyclization gave the title compound.

Example 3. 2-Amino-6,7-bis(4-bromophenyl)-1,9-dimethyl-
imidazol[4,5-g]quinoxaline

2,3-bis(4-bromophenyl)-5-methyl-6-methylaminoquinoxaline was
treated with trifluoroacetic anhydride to give 2,3-bis-(4-bromo
5 phenyl)-5-methyl-6-(N-methyl-N-trifluoroacetyl-amino)quinoxaline
which was then nitrated with fuming nitric acid in trifluoroacetic
anhydride followed by reduction with palladium on charcoal
deprotection with potassium carbonate in methanol, and cyclization
with cyanogen bromide to yield the title compound.

10 Example 4. 6,7-Bis(4-bromophenyl)-2-Methylamino-1,9-
dimethylimidazol-[4,5-g]quinoxaline

Methylation of 2-amino-6,7-bis(4-bromophenyl)-1,9-dimethyl-
imidazol[4,5-g]quinoxaline with methyl iodide in acetone in the
presence of potassium carbonate gave the title compound.

15 6. BIOLOGICAL EVALUATION

It will be appreciated that, in any given series of
compounds, a spectrum of biological activity will be afforded.
In its most preferred embodiments, this invention relates to
tricyclic quinoxaline compounds demonstrating the ability to
20 modulate RTK and CTK activity. The following assays are employed
to select those compounds demonstrating the optimal degree of the
desired activity.

As used herein, the phrase "optimal degree of the desired
activity" refers to the lowest IC50, defined elsewhere herein,

against a PTK related to a particular disorder so as to provide an organism, preferably a human, with a therapeutically effective amount of a compound of this invention at the lowest possible dosage.

5 B. Assay Procedures.

 The following in vitro assays may be used to determine the level of activity and the effect of that activity produced by the compounds of the present invention with respect to specific PTKs. Similar assays can be designed along the same lines for
10 any PK using techniques well known in the art.

 The cellular/catalytic assays described herein are performed in an ELISA format. The general procedure is as follows: a compound is introduced to cells expressing the test kinase, either naturally or recombinantly, for a selected period of time
15 after which, if the test kinase is a receptor, a ligand known to activate the receptor's activity is added. The cells are lysed and the lysate is transferred to the wells of an ELISA plate previously coated with a specific antibody recognizing the substrate of the enzymatic phosphorylation reaction.

20 Non-substrate components of the cell lysate are washed away and the amount of phosphorylation on the substrate is detected with an antibody specifically recognizing phosphotyrosine and is compared with that of control cells that were not contacted with the test compound.

The cellular/biologic assays described herein measure the amount of DNA made in response to activation of a test kinase, which is a general measure of a proliferative response. The general procedure for this assay is as follows: a compound is introduced to cells expressing the test kinase, either naturally or recombinantly, for a selected period of time after which, if the test kinase is a receptor, a ligand known to activate the receptor's activity is added. After incubation at least overnight, a DNA labeling reagent such as Bromodeoxyuridine (BrdU) or 3H-thymidine is added. The amount of labeled DNA is detected with either an anti-BrdU antibody or by measuring radioactivity and is compared to control cells not contacted with a test compound.

1. Cellular/Catalytic Assays

Enzyme linked immunosorbent assays (ELISA) may be used to detect and measure the presence of PTK activity. The ELISA may be conducted according to known protocols which are described in, for example, Voller, et al., 1980, "Enzyme-Linked Immunosorbent Assay," In: Manual of Clinical Immunology, 2d ed., edited by Rose and Friedman, pp 359-371 Am. Soc. Of Microbiology, Washington, D.C.

The disclosed protocol may be adapted for determining activity with respect to a specific PK. The preferred protocols for conducting the ELISA experiments for specific RTKs is

provided below. Adaptation of these protocols for determining a compound's activity for other members of the RTK family, as well as for CTKs, is well within the scope of knowledge of those skilled in the art.

5

a. FLK-1

An ELISA assay is conducted to measure the kinase activity of the FLK-1 receptor and more specifically, the inhibition or activation of TK activity on the FLK-1 receptor. Specifically, the following assay can be conducted to measure
10 kinase activity of the FLK-1 receptor in cells genetically engineered to express Flk-1.

Materials And Methods.

Materials. The following reagents and supplies are used:

- 15 a. Corning 96-well ELISA plates (Corning Catalog No. 25805-96);
- b. Cappel goat anti-rabbit IgG (catalog no. 55641);
- 20 c. PBS (Gibco Catalog No. 450-1300EB);
- d. TBSW Buffer (50 mM Tris (pH 7.2), 150 mM NaCl and 0.1% Tween-20);
- 25 e. Ethanolamine stock (10% ethanolamine (pH 7.0), stored at 4°C);
- f. HNTG buffer (20mM HEPES buffer (pH 7.5), 150mM NaCl, 0.2% Triton X-100, and 10% glycerol);
- 30 g. EDTA (0.5 M (pH 7.0) as a 100X stock);
- h. Sodium orthovanadate (0.5 M as a 100X stock);

- i. Sodium pyrophosphate (0.2 M as a 100X stock);
- j. NUNC 96 well V bottom polypropylene plates (Applied Scientific Catalog No. AS-72092);
- k. NIH3T3 C7#3 Cells (FLK-1 expressing cells);
- l. DMEM with 1X high glucose L-Glutamine (catalog No. 11965-050);
- m. FBS, Gibco (catalog no. 16000-028);
- n. L-glutamine, Gibco (catalog no. 25030-016);
- o. VEGF, PeproTech, Inc. (catalog no. 100-20) (kept as 1 µg/100 µl stock in Milli-Q dH₂O and stored at -20°C;
- p. Affinity purified anti-FLK-1 antiserum;
- q. UB40 monoclonal antibody specific for phosphotyrosine (see, Fendley, et al., 1990, Cancer Research 50:1550-1558);
- r. EIA grade Goat anti-mouse IgG-POD (BioRad catalog no. 172-1011);
- s. 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) solution (100mM citric acid (anhydrous), 250 mM Na₂HPO₄ (pH 4.0), 0.5 mg/ml ABTS (Sigma catalog no. A-1888)), solution should be stored in dark at 4°C until ready for use;
- t. H₂O₂ (30% solution) (Fisher catalog no. H325);
- u. ABTS/H₂O₂ (15ml ABTS solution, 2 µl H₂O₂) prepared 5 minutes before use and left at room temperature;
- v. 0.2 M HCl stock in H₂O;
- w. dimethylsulfoxide (100%) (Sigma Catalog No. D-8418); and
- y. Trypsin-EDTA (Gibco BRL Catalog No. 25200-049).

Protocol. The following protocol can be used for conducting the assay:

1. Coat Corning 96-well ELISA plates with 1.0µg per well Cappel Anti-rabbit IgG antibody in 0.1M Na₂CO₃ pH 9.6.
- 5 Bring final volume to 150 µl per well. Coat plates overnight at 4°C. Plates can be kept up to two weeks when stored at 4°C.
2. Grow cells in Growth media (DMEM, supplemented with 2.0mM L-Glutamine, 10% FBS) in suitable culture dishes until confluent at 37°C, 5% CO₂.
- 10 3. Harvest cells by trypsinization and seed in Corning 25850 polystyrene 96-well round bottom cell plates, 25,000 cells/well in 200µl of growth media.
4. Grow cells at least one day at 37°C, 5% CO₂.
5. Wash cells with D-PBS 1X.
- 15 6. Add 200µl/well of starvation media (DMEM, 2.0mM L-Glutamine, 0.1% FBS). Incubate overnight at 37°C, 5% CO₂.
7. Dilute Compounds 1:20 in polypropylene 96 well plates using starvation media. Dilute dimethylsulfoxide 1:20 for use in control wells.
- 20 8. Remove starvation media from 96 well cell culture plates and add 162 µl of fresh starvation media to each well.
9. Add 18µl of 1:20 diluted Compound dilution (from step 7) to each well plus the 1:20 dimethylsulfoxide dilution to

the control wells (+/- VEGF), for a final dilution of 1:200 after cell stimulation. Final dimethylsulfoxide is 0.5%. Incubate the plate at 37°C, 5% CO₂ for two hours.

10. Remove unbound antibody from ELISA plates by
5 inverting plate to remove liquid. Wash 3 times with TBSW + 0.5% ethanolamine, pH 7.0. Pat the plate on a paper towel to remove excess liquid and bubbles.

11. Block plates with TBSW + 0.5% Ethanolamine, pH 7.0,
150 µl per well. Incubate plate thirty minutes while shaking on
10 a microtiter plate shaker.

12. Wash plate 3 times as described in step 10.

13. Add 0.5µg/well affinity purified anti-FLU-1
polyclonal rabbit antiserum. Bring final volume to 150µl/well
with TBSW + 0.5% ethanolamine pH 7.0. Incubate plate for thirty
15 minutes while shaking.

14. Add 180 µl starvation medium to the cells and
stimulate cells with 20µl/well 10.0mM sodium ortho vanadate and
500 ng/ml VEGF (resulting in a final concentration of 1.0mM
sodium ortho vanadate and 50ng/ml VEGF per well) for eight
20 minutes at 37°C, 5% CO₂. Negative control wells receive only
starvation medium.

15. After eight minutes, media should be removed from
the cells and washed one time with 200µl/well PBS.

16. Lyse cells in 150 μ l/well HNTG while shaking at room temperature for five minutes. HNTG formulation includes sodium ortho vanadate, sodium pyrophosphate and EDTA.

17. Wash ELISA plate three times as described in step 10.

5 18. Transfer cell lysates from the cell plate to ELISA plate and incubate while shaking for two hours. To transfer cell lysate pipette up and down while scrapping the wells.

19. Wash plate three times as described in step 10.

10 20. Incubate ELISA plate with 0.02 μ g/well UB40 in TBSW + 05% ethanolamine. Bring final volume to 150 μ l/well. Incubate while shaking for 30 minutes.

21. Wash plate three times as described in step 10.

15 22. Incubate ELISA plate with 1:10,000 diluted EIA grade goat anti-mouse IgG conjugated horseradish peroxidase in TBSW + 0.5% ethanolamine, pH 7.0. Bring final volume to 150 μ l/well. Incubate while shaking for thirty minutes.

23. Wash plate as described in step 10.

24. Add 100 μ l of ABTS/H₂O₂ solution to well. Incubate ten minutes while shaking.

20 25. Add 100 μ l of 0.2 M HCl for 0.1 M HCl final to stop the color development reaction. Shake 1 minute at room temperature. Remove bubbles with slow stream of air and read the ELISA plate in an ELISA plate reader at 410 nm.

b. HER-2 ELISA

Assay 1: EGF Receptor-HER2 Chimeric
Receptor Assay In Whole Cells.

5 HER2 kinase activity in whole EGFR-NIH3T3 cells are
measured as described below:

Materials and Reagents. The following materials and
reagents can be used to conduct the assay:

- 10 a. EGF: stock concentration: 16.5 ILM; EGF 201, TOYOBO,
Co., Ltd. Japan.
- b. 05-101 (UBI) (a monoclonal antibody recognizing an
EGFR extracellular domain).
- 15 c. Anti-phosphotyrosine antibody (anti-Ptyr)
(polyclonal)(see, Fendley, et al., supra).
- d. Detection antibody: Goat anti-rabbit IgG horse
radish peroxidase conjugate, TAGO, Inc., Burlingame,
20 CA.
- e. TBST buffer:
- | | | |
|----|------------------|--------|
| 25 | Tris-HCl, pH 7.2 | 50 mM |
| | NaCl | 150 mM |
| | Triton X-100 | 0.1 |
- f. HNTG 5X stock:
- | | | |
|----|--------------|--------|
| 30 | HEPES | 0.1 M |
| | NaCl | 0.75 M |
| | Glycerol | 50% |
| | Triton X-100 | 1.0% |
- 35 g. ABTS stock:
- | | | |
|--|----------------------------------|--------|
| | Citric Acid | 100 mM |
| | Na ₂ HPO ₄ | 250 mM |
| | HCl, conc. | 0.5 pM |

ABTS* 0.5mg/ml
* (2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)). Keep solution in dark at 4°C until use.

5 h. Stock reagents of:

EDTA 100 mM pH 7.0
Na₃VO₄ 0.5 M
Na₄(P₂O₇) 0.2 M

10 **Procedure.** The following protocol is used:

A. Pre-coat ELISA Plate

1. Coat ELISA plates (Corning, 96 well, Cat. #25805-96) with 05-101 antibody at 0.5 g per well in PBS, 100 µl final volume/well, and store overnight at 4°C. Coated plates are
15 good for up to 10 days when stored at 4°C.

2. On day of use, remove coating buffer and replace with 100 µl blocking buffer (5% Carnation Instant Non-Fat Dry Milk in PBS). Incubate the plate, shaking, at room temperature (about 23°C to 25°C) for 30 minutes. Just prior to
20 use, remove blocking buffer and wash plate 4 times with TBST buffer.

B. Seeding Cells

1. An NIH3T3 cell line overexpressing a chimERIC receptor containing the EGFR extracellular domain and
25 intracellular HER2 kinase domain can be used for this assay.

2. Choose dishes having 80-90% confluence for the experiment. Trypsinize cells and stop reaction by adding 10%

fetal bovine serum. Suspend cells in DMEM medium (10% CS DMEM medium) and centrifuge once at 1500 rpm, at room temperature for 5 minutes.

3. Resuspend cells in seeding medium (DMEM, 0.5% bovine serum), and count the cells using trypan blue. Viability above 90% is acceptable. Seed cells in DMEM medium (0.5% bovine serum) at a density of 10,000 cells per well, 100 μ l per well, in a 96 well microtiter plate. Incubate seeded cells in 5% CO₂ at 37°C for about 40 hours.

10 C. Assay Procedures

1. Check seeded cells for contamination using an inverted microscope. Dilute drug stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 μ l to a TBST well for a final drug dilution of 1:200 and a final DMSO concentration of 1%. Control
15 wells receive DMSO alone. Incubate in 5% CO₂ at 37°C for two hours.

2. Prepare EGF ligand: dilute stock EGF in DMEM so that upon transfer of 10 μ l dilute EGF (1:12 dilution), 100 nM final concentration is attained.

20 3. Prepare fresh HNTG* sufficient for 100 μ l per well; and place on ice.

HNTG* (10 ml):

HNTG stock 2.0 ml

milli-Q H ₂ O	7.3 ml
EDTA, 100 mM, pH 7.0	0.5 ml
Na ₃ VO ₄ , 0.5 M	0.1 ml
Na ₄ (P ₂ O ₇), 0.2 M	0.1 ml

5 4. After 120 minutes incubation with drug, add prepared SGF ligand to cells, 10 μ l per well, to a final concentration of 100 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

10 5. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 μ l per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TBST as described above.

15 6. With a pipette tip securely fitted to a micropipettor, scrape cells from plate and homogenize cell material by repeatedly aspirating and dispensing the HNTG* lysis buffer. Transfer lysate to a coated, blocked, and washed ELISA plate. Incubate shaking at room temperature for one hour.

20 7. Remove lysate and wash 4 times with TBST. Transfer freshly diluted anti-Ptyr antibody to ELISA plate at 100 μ l per well. Incubate shaking at room temperature for 30 minutes in the presence of the anti-Ptyr antiserum (1:3000 dilution in TBST).

8. Remove the anti-Ptyr antibody and wash 4 times with TBST. Transfer the freshly diluted TAGO anti-rabbit IgG

antibody to the ELISA plate at 100 μ l per well. Incubate shaking at room temperature for 30 minutes (anti-rabbit IgG antibody: 1:3000 dilution in TBST).

9. Remove TAGO detection antibody and wash 4 times with TBST. Transfer freshly prepared ABTS/ H_2O_2 solution to ELISA plate, 100 μ l per well. Incubate shaking at room temperature for 20 minutes. (ABTS/ H_2O_2 solution: 1.0 μ l 30% H_2O_2 in 10 ml ABTS stock).

10. Stop reaction by adding 50 μ l 5N H_2SO_4 (optional), and determine O.D. at 410 nm.

11. The maximal phosphotyrosine signal is determined by subtracting the value of the negative controls from the positive controls. The percent inhibition of phosphotyrosine content for extract-containing wells is then calculated, after subtraction of the negative controls.

c. PDGF-R ELISA

All cell culture media, glutamine, and fetal bovine serum can be purchased from Gibco Life Technologies (Grand Island, NY) unless otherwise specified. All cells are grown in a humid atmosphere of 90-95% air and 5-10% CO_2 at 37°C. All cell lines are routinely subcultured twice a week and are negative for mycoplasma as determined by the Mycotect method (Gibco).

For ELISA assays, cells (U1242, obtained from Joseph Schlessinger, NYU) are grown to 80-90% confluency in growth medium (MEM with 10% FBS, NEAA, 1 mM NaPyr and 2 mM GLN) and seeded in 96-well tissue culture plates in 0.5% serum at 25,000 to 30,000 cells per well. After overnight incubation in 0.5% serum-containing medium, cells are changed to serum-free medium and treated with test compound for 2 hr in a 5% CO₂, 37°C incubator. Cells are then stimulated with ligand for 5-10 minute followed by lysis with HNTG (20 mM Hepes, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 5 mM Na₃VO₄, 0.2% Triton X-100, and 2 mM NaPyr). Cell lysates (0.5 mg/well in PBS) are transferred to ELISA plates previously coated with receptor-specific antibody and which had been blocked with 5% milk in TBST (50 mM Tris-HCl pH 7.2, 150 mM NaCl and 0.1% Triton X-100) at room temperature for 30 min. Lysates are incubated with shaking for 1 hour at room temperature. The plates are washed with TBST four times and then incubated with polyclonal anti-phosphotyrosine antibody at room temperature for 30 minutes. Excess anti-phosphotyrosine antibody is removed by rinsing the plate with TBST four times. Goat anti-rabbit IgG antibody is added to the ELISA plate for 30 min at room temperature followed by rinsing with TBST four more times. ABTS (100 mM citric acid, 250 mM Na₂HPO₄ and 0.5 mg/mL 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) plus H₂O₂ (1.2 mL 30% H₂O₂ to 10 ml ABTS) is added to the ELISA plates to

start color development. Absorbance at 410 nm with a reference wavelength of 630 nm is recorded about 15 to 30 min after ABTS addition.

d. IGF-I RECEPTOR ELISA

The following protocol may be used to measure phosphotyrosine level on IGF-I receptor, which indicates IGF-I receptor tyrosine kinase activity.

Materials And Reagents. The following materials and reagents are used:

a. The cell line used in this assay is 3T3/IGF-1R, a cell line genetically engineered to overexpresses IGF-1 receptor.

b. NIH3T3/IGF-1R is grown in an incubator with 5% CO₂ at 37°C. The growth media is DMEM + 10% FBS (heat inactivated) + 2mM L-glutamine.

c. Affinity purified anti-IGF-1R antibody 17-69.

d. D-PBS:

KH ₂ PO ₄	0.20 g/l
K ₂ HPO ₄	2.16 g/l
KCl	0.20 g/l
NaCl	8.00 g/l (pH 7.2)

e. Blocking Buffer: TBST plus 5% Milk (Carnation Instant Non-Fat Dry Milk).

f. TBST buffer:

Tris-HCl	50 mM
NaCl	150mM (pH 7.2/HCl 10N)
Triton X-100	0.1%

Stock solution of TBS (10X) is prepared, and Triton X-100 is added to the buffer during dilution.

g. HNTG buffer:

HEPES 20 mM
NaCl 150 mM (pH 7.2/HCl 1N)
Glycerol 10%
Triton X-100 0.2%

Stock solution (5X) is prepared and kept at 4°C.

h. EDTA/HCl: 0.5 M pH 7.0 (NaOH) as 100X stock.

i. Na_3VO_4 : 0.5 M as 100X stock and aliquots are kept in -80°C.

j. $\text{Na}_4\text{P}_2\text{O}_7$: 0.2 M as 100X stock.

k. Insulin-like growth factor-1 from Promega (Cat# G5111).

l. Rabbit polyclonal anti-phosphotyrosine antiserum.

m. Goat anti-rabbit IgG, POD conjugate (detection antibody), Tago (Cat. No. 4520, Lot No. 1802): Tago, Inc., Burlingame, CA.

n. ABTS (2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)) solution:

Citric acid 100 mM
 Na_2HPO_4 250 mM (pH 4.0/1 N HCl)
ABTS 0.5 mg/ml

ABTS solution should be kept in dark and 4°C. The solution should be discarded when it turns green.

o. Hydrogen Peroxide: 30% solution is kept in the dark and at 4°C.

Procedure. All the following steps are conducted at room temperature unless it is specifically indicated. All ELISA plate washings are performed by rinsing the plate with tap water three times, followed by one TBST rinse. Pat plate dry with paper

towels.

A. Cell Seeding:

1. The cells, grown in tissue culture dish (Corning 25020-100) to 80-90% confluence, are harvested with
5 Trypsin-EDTA (0.25%, 0.5 ml/D-100, GIBCO).
2. Resuspend the cells in fresh DMEM + 10% FBS + 2mM L-Glutamine, and transfer to 96-well tissue culture plate (Corning, 25806-96) at 20,000 cells/well (100 μ l/well). Incubate
for 1 day then replace medium to serum-free medium (90/ μ l) and
10 incubate in 5% CO₂ and 37°C overnight.

B. ELISA Plate Coating and Blocking:

1. Coat the ELISA plate (Corning 25805-96) with Anti-IGF-1R Antibody at 0.5 μ g/well in 100 μ l PBS at least 2
15 hours.
2. Remove the coating solution, and replace with 100 μ l Blocking Buffer, and shake for 30 minutes. Remove the blocking buffer and wash the plate just before adding lysate.

C. Assay Procedures:

1. The drugs are tested in serum-free condition.
2. Dilute drug stock (in 100% DMSO) 1:10 with DMEM.
in 96-well poly-propylene plate, and transfer 10 μ l/well of this

solution to the cells to achieve final drug dilution 1:100, and final DMSO concentration of 1.0%. Incubate the cells in 5% CO₂ at 37°C for 2 hours.

3. Prepare fresh cell lysis buffer (HNTG*)

5	HNTG	2 ml
	EDTA	0.1 ml
	Na ₃ VO ₄	0.1 ml
	Na ₄ (P ₂ O ₇)	0.1 ml
	H ₂ O	7.3 ml

10 4. After drug incubation for two hours, transfer 10 µl/well of 200nM IGF-1 Ligand in PBS to the cells (Final Conc. = 20 nM), and incubate at 5% CO₂ at 37°C for 10 minutes.

15 5. Remove media and add 100µl/well HNTG* and shake for 10 minutes. Look at cells under microscope to see if they are adequately lysed.

6. Use a 12-channel pipette to scrape the cells from the plate, and homogenize the lysate by repeated aspiration and dispensing. Transfer all the lysate to the antibody coated ELISA plate, and shake for 1 hour..

20 7. Remove the lysate, wash the plate, transfer anti-pTyr (1:3,000 with TBST) 100 µl/well, and shake for 30 minutes.

8. Remove anti-pTyr, wash the plate, transfer TAGO

(1:3,000 with TBST) 100 μ l/well, and shake for 30 minutes.

9. Remove detection antibody, wash the plate, and transfer fresh ABTS/H₂O₂ (1.2 μ l H₂O₂ to 10 ml ABTS) 100 μ l/well to the plate to start color development.

5 10. Measure OD at 410 nm with a reference wavelength of 630 nm in Dynatec MR5000.

e. EGF Receptor ELISA

EGF Receptor kinase activity in cells

10 genetically engineered to express human EGF-R can be measured as described below:

Materials and Reagents. The following materials and reagents are used

- 15 a. EGF Ligand: stock concentration = 16.5 μ M; EGF 201, TOYOKO, Co., Ltd. Japan.
- b. 05-101 (UBI) (a monoclonal antibody recognizing an EGFR extracellular domain).
- 20 c. Anti-phosphotyrosine antibody (anti-Ptyr) (polyclonal).
- d. Detection antibody: Goat anti-rabbit IgG horse radish peroxidase conjugate, TAGO, Inc., Burlingame, CA.
- 25 e. TBST buffer:
- | | |
|------------------------------------|--------|
| Tris-HCl, pH 7 | 50 mM |
| NaCl | 150 mM |
| 30 Triton X-100 | 0.1 |
- f. HNTG 5X stock:
- | | |
|-------|-------|
| HEPES | 0.1 M |
|-------|-------|

NaCl	0.75 M
Glycerol	50
Triton X-100	1.0%

5 g. ABTS stock:

Citric Acid	100 mM
Na ₂ HPO ₄	250 mM
HCl, conc.	4.0 pH
ABTS*	0.5 mg/ml

10 Keep solution in dark at 4°C until used.

15 h. Stock reagents of:

EDTA 100 mM pH 7.0
Na₃VO₄ 0.5 M
Na₄(P₂O₇) 0.2 M

20 Procedure. The following protocol is used:

A. Pre-coat ELISA Plate

1. Coat ELISA plates (Corning, 96 well, Cat.

#25805-96) with 05-101 antibody at 0.5 µg per well in PBS, 150 µl
final volume/well, and store overnight at 4°C. Coated plates are
25 good for up to 10 days when stored at 4°C.

2. On day of use, remove coating buffer and
replace with blocking buffer (5% Carnation Instant NonFat Dry
Milk in PBS). Incubate the plate, shaking, at room temperature
(about 23°C to 25°C) for 30 minutes. Just prior to use, remove
30 blocking buffer and wash plate 4 times with TBST buffer.

B. Seeding Cells

1. NIH 3T3/C7 cell line (Honegger, et al., Cell
51:199-209, 1987) can be use for this assay.

2. Choose dishes having 80-90% confluence for the experiment. Trypsinize cells and stop reaction by adding 10% CS DMEM medium. Suspend cells in DMEM medium (10% CS DMEM medium) and centrifuge once at 1000 rpm at room temperature for 5 minutes.

3. Resuspend cells in seeding medium (DMEM, 0.5% bovine serum), and count the cells using trypan blue. Viability above 90% is acceptable. Seed cells in DMEM medium (0.5% bovine serum) at a density of 10,000 cells per well, 100 μ l per well, in a 96 well microtiter plate. Incubate seeded cells in 5% CO₂ at 37°C for about 40 hours.

C. Assay Procedures.

1. Check seeded cells for contamination using an inverted microscope. Dilute drug stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 μ l to a test well for a final drug dilution of 1:200 and a final DMSO concentration of 1%. Control wells receive DMSO alone. Incubate in 5% CO₂ at 37°C for one hour.

2. Prepare EGF ligand: dilute stock EGF in DMEM so that upon transfer of 10 μ l dilute EGF (1:12 dilution), 25 nM final concentration is attained.

3. Prepare fresh 10 ml HNTG* sufficient for 100 μ l per well wherein HNTG* comprises: HNTG stock (2.0 ml), milli-Q

H₂O (7.3 ml), EDTA, 100 mM, pH 7.0 (0.5 ml), Na₃VO₄ 0.5 M (0.1 ml) and Na₄(P₂O₇), 0.2 M (0.1 ml).

4. Place on ice.

5. After two hours incubation with drug, add
5 prepared EGF ligand to cells, 10 µl per well, to yield a final concentration of 25 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

6. Remove drug, EGF, and DMEM. Wash cells twice
with PBS. Transfer HNTG* to cells, 100 µl per well. Place on
10 ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TBST as described above.

7. With a pipette tip securely fitted to a micropipettor, scrape cells from plate and homogenize cell material by repeatedly aspirating and dispensing the HNTG* lysis
15 buffer. Transfer lysate to a coated, blocked, and washed ELISA plate. Incubate shaking at room temperature for one hour.

8. Remove lysate and wash 4 times with TBST. Transfer freshly diluted anti-Ptyr antibody to ELISA plate at 100 µl per well. Incubate shaking at room temperature for 30 minutes
20 in the presence of the anti-Ptyr antiserum (1:3000 dilution in TBST).

9. Remove the anti-Ptyr antibody and wash 4 times with TBST. Transfer the freshly diluted TAGO 30 anti-rabbit IgG

antibody to the ELISA plate at 100 μ l per well. Incubate shaking at room temperature for 30 minutes (anti-rabbit IgG antibody: 1:3000 dilution in TBST).

10. Remove detection antibody and wash 4 times with
5 TBST. Transfer freshly prepared ABTS/ H_2O_2 solution to ELISA plate, 100 μ l per well. Incubate at room temperature for 20 minutes. ABTS/ H_2O_2 solution: 1.2 μ l 30% H_2O_2 in 10 ml ABTS stock.

11. Stop reaction by adding 50 μ l 5N H_2SO_4 (optional), and determine O.D. at 410 nm.

10 12. The maximal phosphotyrosine signal is determined by subtracting the value of the negative controls from the positive controls. The percent inhibition of phosphotyrosine content for extract-containing wells is then calculated, after subtraction of the negative controls.

15

f. Met Autophosphorylation Assay - ELISA

This assay determines Met tyrosine kinase activity by analyzing Met protein tyrosine kinase levels on the Met receptor.

1. Reagents

20 a. HNTG (5X stock solution): Dissolve 23.83 g HEPES and 43.83 g NaCl in about 350 ml dH_2O . Adjust pH to 7.2 with HCl or NaOH, add 500 ml glycerol and 10 ml Triton X-100, mix, add dH_2O to 1 L total volume. To

make 1 L of 1X working solution add 200 ml 5X stock solution to 800 ml dH₂O, check and adjust pH as necessary, store at 4°C.

- 5 b. PBS (Dulbecco's Phosphate-Buffered Saline), Gibco
Cat. # 450-1300EB (1X solution).
- c. Blocking Buffer: in 500 ml dH₂O place 100 g BSA,
12.1 g Tris-pH7.5, 58.44 g NaCl and 10 ml Tween-20,
dilute to 1 L total volume.
- 10 d. Kinase Buffer: To 500 ml dH₂O add 12.1 g TRIS pH7.2,
58.4 g NaCl, 40.7 g MgCl₂ and 1.9 g EGTA; bring to 1
L total volume with dH₂O.
- e. PMSF (Phenylmethanesulfonyl fluoride), Sigma Cat. #
P-7626, to 435.5 mg, add 100% ethanol to 25 ml total
volume, vortex.
- 15 f. ATP (Bacterial Source), Sigma Cat. # A-7699, store
powder at -20°C; to make up solution for use,
dissolve 3.31 mg in 1 ml dH₂O.
- g. RC-20H HRPO Conjugated Anti-Phosphotyrosine,
Transduction Laboratories Cat. # E120H.
- 20 h. Pierce 1-Step (TM) Turbo TMB-ELISA (3,3',5,5'-
tetramethylbenzidine, Pierce Cat. # 34022.
- i. H₂SO₄, add 1 ml conc. (18N) to 35 ml dH₂O.
- j. TRIS HCL, Fischer Cat. # BP152-5; to 121.14 g of
material, add 600 ml MilliQ H₂O, adjust pH to 7.5

(or 7.2) with HCl , bring volume to 1 L with MilliQ H₂O.

k. NaCl, Fischer Cat. # S271-10, make up 5M solution.

l. Tween-20, Fischer Cat. # S337-500.

5 m. Na₃VO₄, Fischer Cat. # S454-50, to 1.8 g material add 80 ml MilliQ H₂O, adjust pH to 10.0 with HCl or NaOH, boil in microwave, cool, check pH, repeat procedure until pH stable at 10.0, add MilliQ H₂O to 100 ml total volume, make 1 ml aliquots and store at
10 -80°C.

n. MgCl₂, Fischer Cat. # M33-500, make up 1M solution.

o. HEPES, Fischer Cat. # BP310-500, to 200 ml MilliQ H₂O, add 59.6 g material, adjust pH to 7.5, bring volume to 250 ml total, sterile filter.

15 p. Albumin, Bovine (BSA), Sigma Cat. # A-4503, to 30 grams material add sterile distilled water to make total volume of 300 ml, store at 4°C.

q. TBST Buffer: to approx. 900 ml dH₂O in a 1 L graduated cylinder add 6.057 g TRIS and 8.766 g
20 NaCl, when dissolved, adjust pH to 7.2 with HCl, add 1.0 ml Triton X-100 and bring to 1 L total volume with dH₂O.

r. Goat Affinity purified antibody Rabbit IgG (whole molecule), Cappel Cat. # 55641.

- s. Anti h-Met (C-28) rabbit polyclonal IgG antibody,
Santa Cruz Chemical Cat. # SC-161.
- t. Transiently Transfected EGFR/Met chimeric cells
(EMR) (Komada, et al., Oncogene, 8:2381-2390 (1993)).
- 5 u. Sodium Carbonate Buffer, (Na_2CO_3 , Fischer Cat. # S495):
to 10.6 g material add 800 ml MilliQ H_2O , when
dissolved adjust pH to 9.6 with NaOH, bring up to 1 L
total volume with MilliQ H_2O , filter, store at 4°C.

2. Procedure

10 All of the following steps are conducted at room
temperature unless it is specifically indicated otherwise. All
ELISA plate washing is by rinsing 4X with TBST.

A. EMR Lysis

15 This procedure can be performed the night before or
immediately prior to the start of receptor capture.

1. Quick thaw lysates in a 37°C waterbath with a
swirling motion until the last crystals
disappear.
2. Lyse cell pellet with 1X HNTG containing 1 mM
20 PMSF. Use 3 ml of HNTG per 15 cm dish of
cells. Add 1/2 the calculated HNTG volume,
vortex the tube for 1 min., add the remaining
amount of HNTG, vortex for another min.
3. Balance tubes, centrifuge at 10,000x g for 10

min at 4°C.

4. Pool supernatants, remove an aliquot for protein determination.
5. Quick freeze pooled sample in dry ice/ethanol bath. This step is performed regardless of whether lysate will be stored overnight or used immediately following protein determination.
6. Perform protein determination using standard bicinchoninic acid (BCA) method (BCA Assay Reagent Kit from Pierce Chemical Cat. # 23225).

B. ELISA Procedure

1. Coat Corning 96 well ELISA plates with 5 µg per well Goat anti-Rabbit antibody in Carbonate Buffer for a total well volume of 50 µl. Store overnight at 4°C.
2. Remove unbound Goat anti-rabbit antibody by inverting plate to remove liquid.
3. Add 150 µl of Blocking Buffer to each well. Incubate for 30 min. at room temperature with shaking.
4. Wash 4X with TBST. Pat plate on a paper towel to remove excess liquid and bubbles.

5. Add 1µg per well of Rabbit anti-Met antibody
diluted in TBST for a total well volume of 100
µl.
6. Dilute lysate in HNTG (90 µg lysate/100µl)
- 5 7. Add 100 µl of diluted lysate to each well.
Shake at room temperature for 60 min.
8. Wash 4X with TBST. Pat on paper towel to
remove excess liquid and bubbles.
9. Add 50 µl of 1X lysate buffer per well.
- 10 10. Dilute compounds/extracts 1:10 in 1X Kinase
Buffer in a polypropylene 96 well plate.
11. Transfer 5.5 µl of diluted drug to ELISA plate
wells. Incubate at room temperature with
shaking for 20 min.
- 15 12. Add 5.5 µl of 60 µM ATP solution per well.
Negative controls do not receive any ATP.
Incubate at room temperature for 90 min., with
shaking.
13. Wash 4X with TBST. Pat plate on paper towel to
remove excess liquid and bubbles.
- 20 14. Add 100 µl per well of RC20 (1:3000 dilution in
Blocking Buffer). Incubate 30 min. at room
temperature with shaking.

15. Wash 4X with TBST. Pat plate on paper towel to remove excess liquid and bubbles.
16. Add 100 μ l per well of Turbo-TMB. Incubate with shaking for 30-60 min.
17. Add 100 μ l per well of 1M H_2SO_4 to stop reaction.
18. Read assay on Dynatech MR7000 ELISA reader.
Test Filter = 450 nm, reference filter = 410 nm.

g. Biochemical src assay - ELISA

This assay is used to determine src protein kinase activity measuring phosphorylation of a biotinylated peptide as the readout.

1. Materials and Reagents:

- a. Yeast transformed with src from Courtneidge Laboratory (Sugen, Inc., Redwood City, California).
- b. Cell lysates: Yeast cells expressing src are pelleted, washed once with water, re-pelleted and stored at $-80^{\circ}C$ until use.
- c. N-terminus biotinylated EEEYEEYEEEEEEEEEEY is prepared by standard procedures well known to those skilled in the art.
- d. DMSO: Sigma, St. Louis, MO.

- e. 96 Well ELISA Plate: Corning 96 Well Easy Wash,
Modified flat Bottom Plate, Corning Cat. #25805-96.
- f. NUNC 96-well V-bottom polypropylene plates for
dilution of compounds: Applied Scientific Cat. # A-
5 72092.
- g. Vecastain ELITE ABC reagent: Vector, Burlingame,
CA.
- h. Anti-src (327) mab: Schizosaccharomyces Pombe is
used to express recombinant Src (Superti-Furga, et
10 al., EMBO J., 12:2625-2634; Superti-Furga, et al.,
Nature Biochem., 14:600-605). S. Pombe strain SP200
(h-s leul.32 ura4 ade210) is grown as described and
transformations are pRSP expression plasmids are
done by the lithium acetate method (Superti-Furga,
15 supra). Cells are grown in the presence of 1 μ M
thiamine to repress expression from the nmt1
promoter or in the absence of thiamine to induce
expression.
- i. Monoclonal anti-phosphotyrosine, UBI 05-321 (UB40
20 may be used instead).
- j. Turbo TMB-ELISA peroxidase substrate: Pierce
Chemical.

2. Buffer Solutions:

- a. PBS (Dulbecco's Phosphate-Buffered Saline): GIBCO

PBS, GIBCO Cat. # 450-1300EB.

b. Blocking Buffer: 5% Non-fat milk (Carnation) in PBS.

c Carbonate Buffer: Na_2CO_3 from Fischer, Cat. # S495,
make up 100 mM stock solution.

5 d. Kinase Buffer: 1.0 ml (from 1M stock solution)
 MgCl_2 ; 0.2 ml (from a 1M stock solution) MnCl_2 ; 0.2
ml (from a 1M stock solution) DTT; 5.0 ml (from a
1M stock solution) HEPES; 0.1 ml TX-100; bring to 10
ml total volume with MilliQ H_2O .

10 e. Lysis Buffer: 5.0 HEPES (from 1M stock solution.);
2.74 ml NaCl (from 5M stock solution); 10 ml
glycerol; 1.0 ml TX-100; 0.4 ml EDTA (from a 100 mM
stock solution); 1.0 ml PMSF (from a 100 mM stock
solution); 0.1 ml Na_3VO_4 (from a 0.1 M stock
15 solution); bring to 100 ml total volume with MilliQ
 H_2O .

f. ATP: Sigma Cat. # A-7699, make up 10 mM stock
solution (5.51 mg/ml).

20 g TRIS-HCl: Fischer Cat. # BP 152-5, to 600 ml MilliQ
 H_2O add 121.14 g material, adjust pH to 7.5 with
HCl, bring to 1 L total volume with MilliQ H_2O .

h. NaCl: Fischer Cat. # S271-10, Make up 5M stock
solution with MilliQ H_2O .

i. Na_3VO_4 : Fischer Cat. # S454-50; to 80 ml MilliQ H_2O ,

add 1.8 g material; adjust pH to 10.0 with HCl or NaOH;
boil in a microwave; cool; check pH, repeat pH
adjustment until pH remains stable after
heating/cooling cycle; bring to 100 ml total volume
with MilliQ H₂O; make 1 ml aliquots and store at -80°C.

j. MgCl₂: Fischer Cat. # M33-500, make up 1M stock
solution with MilliQ H₂O.

k. HEPES: Fischer Cat. # BP 310-500; too 200 ml MilliQ
H₂O, add 59.6 g material, adjust pH to 7.5, bring to
250 ml total volume with MilliQ H₂O, sterile filter
(1M stock solution).

l. TBST Buffer: TBST Buffer: To 900 ml dH₂O add 6.057
g TRIS and 8.766 g NaCl; adjust pH to 7.2 with HCl,
add 1.0 ml Triton-X100; bring to 1 L total volume
with dH₂O.

m. MnCl₂: Fischer Cat. # M87-100, make up 1M stock
solution with MilliQ H₂O.

n. DTT: Fischer Cat. # BP172-5.

o. TBS (TRIS Buffered Saline): to 900 ml MilliQ H₂O add
6.057 g TRIS and 8.777 g NaCl; bring to 1 L total
volume with MilliQ H₂O.

p. Kinase Reaction Mixture: Amount per assay plate
(100 wells): 1.0 ml Kinase Buffer, 200 µg GST-ζ ,
bring to final volume of 8.0 ml with MilliQ H₂O.

q. Biotin labeled EEEYEEYEEYEEYEEY: Make peptide stock solution (1mM, 2.98 mg/ml) in water fresh just before use.

5 r. Vectastain ELITE ABC reagent: To prepare 14 ml of working reagent, add 1 drop of reagent A to 15 ml TBST and invert tube several times to mix. Then add 1 drop of reagent B. Put tube on orbital shaker at room temperature and mix for 30 minutes.

10 3. Procedures:

a. Preparation of src coated ELISA plate.

1. Coat ELISA plate with 0.5µg/well anti-src mab in 100 µl of pH 9.6 sodium carbonate buffer at 4°C overnight.
- 15 2. Wash wells once with PBS.
3. Block plate with 0.15 ml 5% milk in PBS for 30 min. at room temperature.
4. Wash plate 5X with PBS.
- 20 5. Add 10 µg/well of src transformed yeast lysates diluted in Lysis Buffer (0.1 ml total volume per well). (Amount of lysate may vary between batches.) Shake plate for 20 minutes at room temperature.

b. Preparation of phosphotyrosine antibody-coated ELISA plate.

1. 4G10 plate: coat 0.5 μ g/well 4G10 in 100 μ l PBS overnight at 4°C and block with 150 μ l of 5% milk in PBS for 30 minutes at room temperature.

c. Kinase assay procedure.

1. Remove unbound proteins from step 1-7, above, and wash plates 5X with PBS.
2. Add 0.08 ml Kinase Reaction Mixture per well (containing 10 μ l of 10X Kinase Buffer and 10 μ M (final concentration) biotin-EEEEYEEYEEEEYEEEEY per well diluted in water.
3. Add 10 μ l of compound diluted in water containing 10% DMSO and pre-incubate for 15 minutes at room temperature.
4. Start kinase reaction by adding 10 μ l/well of 0.05 mM ATP in water (5 μ M ATP final).
5. Shake ELISA plate for 15 min. at room temperature.
6. Stop kinase reaction by adding 10 μ l of 0.5 M EDTA per well.
7. Transfer 90 μ l supernatant to a blocked 4G10 coated ELISA plate from section B, above.

8. Incubate for 30 min. while shaking at room temperature.
9. Wash plate 5X with TBST.
10. Incubate with Vectastain ELITE ABC reagent (100
5 μ l/well) for 30 min. at room temperature.
11. Wash the wells 5X with TBST.
12. Develop with Turbo TMB.

h. Biochemical lck Assay - ELISA

10 This assay is used to determine lck protein kinase activities measuring phosphorylation of GST- ζ as the readout.

1. Materials and Reagents:

- a. Yeast transformed with lck. Schizosaccharomyces Pombe is used to express recombinant Lck (Superti-Furga, et al., EMBO J, 12:2625-2634; Superti-Furga, et al., Nature Biotech., 14:600-605). S. Pombe strain SP200 (h-s leul.32 ura4 ade210) is grown as described and transformations with pRSP expression plasmids are done by the lithium acetate method (Superti-Furga, supra). Cells are grown in the
15 presence of 1 μ M thiamine to induce expression.
- b. Cell lysates: Yeast cells expressing lck are
20 pelleted, washed once in water, re-pelleted and

stored frozen at -80°C until use.

- c. GST- ζ : DNA encoding for GST- ζ fusion protein for expression in bacteria obtained from Arthur Weiss of the Howard Hughes Medical Institute at the University of California, San Francisco. Transformed bacteria are grown overnight while shaking at 25°C.

GST- ζ is purified by glutathione affinity chromatography, Pharmacia, Alameda, CA.

- d. DMSO: Sigma, St. Louis, MO.

- e. 96-Well ELISA plate: Corning 96 Well Easy Wash, Modified Flat Bottom Plate, Corning Cat. #25805-96.

- f. NUNC 96-well V-bottom polypropylene plates for dilution of compounds: Applied Scientific Cat. # AS-72092.

- g. Purified Rabbit anti-GST antiserum: Amrad Corporation (Australia) Cat. #90001605.

- h. Goat anti-Rabbit-IgG-HRP: Amersham Cat. # V010301.

- i. Sheep anti-mouse IgG (H+L): Jackson Labs Cat. # 5215-005-003.

- j. Anti-Lck (3A5) mab: Santa Cruz Biotechnology Cat # sc-433.

- k. Monoclonal anti-phosphotyrosine UBI 05-321 (UB40 may be used instead).

2. Buffer solutions:

- a. PBS (Dulbecco's Phosphate-Buffered Saline) 1X
solution: GIBCO PBS, GIBCO Cat. # 450-1300EB.
- 5 b. Blocking Buffer: 100 g. BSA, 12.1 g. TRIS-pH7.5,
58.44 g NaCl, 10 ml Tween-20, bring up to 1 L total
volume with MilliQ H₂O.
- c. Carbonate Buffer: Na₂CO₃ from Fischer, Cat. # S495;
make up 100 mM solution with MilliQ H₂O.
- 10 d. Kinase Buffer: 1.0 ml (from 1M stock solution)
MgCl₂; 0.2 ml (from a 1M stock solution) MnCl₂; 0.2
ml (from a 1M stock solution) DTT; 5.0 ml (from a
1M stock solution) HEPES; 0.1 ml TX-100; bring to 10
ml total volume with MilliQ H₂O.
- 15 e. Lysis Buffer: 5.0 HEPES (from 1M stock solution.);
2.74 ml NaCl (from 5M stock solution); 10 ml
glycerol; 1.0 ml TX-100; 0.4 ml EDTA (from a 100 mM
stock solution); 1.0 ml PMSF (from a 100 mM stock
solution); 0.1 ml Na₃VO₄ (from a 0.1 M stock
20 solution); bring to 100 ml total volume with MilliQ
H₂O.
- f. ATP: Sigma Cat. # A-7699, make up 10 mM stock
solution (5.51 mg/ml).
- g TRIS-HCl: Fischer Cat. # BP 152-5, to 600 ml MilliQ

H₂O add 121.14 g material, adjust pH to 7.5 with HCl, bring to 1 L total volume with MilliQ H₂O.

h. NaCl: Fischer Cat. # S271-10, Make up 5M stock solution with MilliQ H₂O.

5 i. Na₃VO₄: Fischer Cat. # S454-50; to 80 ml MilliQ H₂O, add 1.8 g material; adjust pH to 10.0 with HCl or NaOH; boil in a microwave; cool; check pH, repeat pH adjustment until pH remains stable after heating/cooling cycle; bring to 100 ml total volume
10 with MilliQ H₂O; make 1 ml aliquots and store at -80°C.

j. MgCl₂: Fischer Cat. # M33-500, make up 1M stock solution with MilliQ H₂O.

k. HEPES: Fischer Cat. # BP 310-500; to 200 ml MilliQ H₂O, add 59.6 g material, adjust pH to 7.5, bring to
15 250 ml total volume with MilliQ H₂O, sterile filter (1M stock solution).

l. Albumin, Bovine (BSA), Sigma Cat. # A4503; to 150 ml MilliQ H₂O add 30 g material, bring 300 ml total volume with MilliQ H₂O, filter through 0.22 µm
20 filter, store at 4°C.

m. TBST Buffer: To 900 ml dH₂O add 6.057 g TRIS and 8.766 g NaCl; adjust pH to 7.2 with HCl, add 1.0 ml Triton-X100; bring to 1 L total volume with dH₂O.

n. MnCl₂: Fischer Cat. # M87-100, make up 1M stock

solution with MilliQ H₂O.

o. DTT; Fischer Cat. # BP172-5.

p. TBS (TRIS Buffered Saline): to 900 ml MilliQ H₂O
add 6.057 g TRIS and 8.777 g NaCl; bring to 1 L
total volume with MilliQ H₂O.

q Kinase Reaction Mixture: Amount per assay plate
(100 wells): 1.0 ml Kinase Buffer, 200 µg GST-ζ ,
bring to final volume of 8.0 ml with MilliQ H₂O.

2. Procedures:

a. Preparation of Lck coated ELISA plate.

1. Coat 2.0 µg/well Sheep anti-mouse IgG in 100 µl
of pH 9.6 sodium carbonate buffer at 4°C overnight.

2. Wash well once with PBS.

3. Block plate with 0.15 ml of blocking Buffer for
30 min. at room temp.

4. Wash plate 5X with PBS.

5. Add 0.5 µg/well of anti-lck (mab 3A5) in 0.1 ml
PBS at room temperature for 1-2 hours.

6. Wash plate 5X with PBS.

7. Add 20 µg/well of lck transformed yeast lysates
diluted in Lysis Buffer (0.1 ml total volume per well). (Amount
of lysate may vary between batches) Shake plate at 4°C overnight
to prevent loss of activity.

b. Preparation of phosphotyrosine antibody-coated ELISA plate.

1. UB40 plate: 1.0 μg /well UB40 in 100 μl of PBS overnight at 4°C and block with 150 μl of Blocking Buffer for at least 1 hour.

c. Kinase assay procedure.

1. Remove unbound proteins from step 1-7, above, and wash plates 5X with PBS.

2. Add 0.08 ml Kinase Reaction Mixture per well (containing 10 μl of 10X Kinase Buffer and 2 μg GST- ζ per well diluted with water).

3. Add 10 μl of compound diluted in water containing 10% DMSO and pre-incubate for 15 minutes at room temperature.

4. Start kinase reaction by adding 10 μl /well of 0.1 mM ATP in water (10 μM ATP final).

5. Shake ELISA plate for 60 min. at room temperature.

6. Stop kinase reaction by adding 10 μl of 0.5 M EDTA per well.

7. Transfer 90 μl supernatant to a blocked 4G10 coated ELISA plate from section B, above.

8. Incubate while shaking for 30 min. at room

temperature.

9. Wash plate 5X with TBST.

10. Incubate with Rabbit anti-GST antibody at
1:5000 dilution in 100 μ l TBST for 30 min. at room temperature.

5 11. Wash the wells 5X with TBST.

12. Incubate with Goat anti-Rabbit-IgG-HRP at
1:20,000 dilution in 100 μ l of TBST for 30 min. at room
temperature.

13. Wash the wells 5X with TBST.

10 14. Develop with Turbo TMB.

1. ASSAY MEASURING PHOSPHORYLATING FUNCTION OF RAF

The following assay reports the amount of RAF-catalyzed
15 phosphorylation of its target protein MEK as well as MEK's target
MAPK. The RAF gene sequence is described in Bonner et al., 1985,
Molec. Cell. Biol. 5: 1400-1407, and is readily accessible in
multiple gene sequence data banks. Construction of the nucleic
acid vector and cell lines utilized for this portion of the
20 invention are fully described in Morrison et al., 1988, Proc.
Natl. Acad. Sci. USA 85: 8855-8859.

Materials and Reagents

1. *Sf9* (*Spodoptera frugiperda*) cells; GIBCO-BRL,

Gaithersburg, MD.

2. RIPA buffer: 20 mM Tris/HCl pH 7.4, 137 mM NaCl, 10% glycerol, 1 mM PMSF, 5 mg/L Aprotinin, 0.5 % Triton X-100;

3. Thioredoxin-MEK fusion protein (T-MEK): T-MEK
5 expression and purification by affinity chromatography are performed according to the manufacturer's procedures. Catalog# K 350-01 and R 350-40, Invitrogen Corp., San Diego, CA

4. His-MAPK (ERK 2); His-tagged MAPK is expressed in XL1 Blue cells transformed with pUC18 vector encoding His-MAPK.

10 His-MAPK is purified by Ni-affinity chromatography. Cat# 27-4949-01, Pharmacia, Alameda, CA, as described herein.

5. Sheep anti mouse IgG: Jackson laboratories, West Grove, PA. Catalog, # 515-006-008, Lot# 28563

6. RAF-1 protein kinase specific antibody: URP2653 from
15 UBI.

7. Coating buffer: PBS; phosphate buffered saline, GIBCO-BRL, Gaithersburg, MD.

8. Wash buffer: TBST - 50 mM Tris/HCL pH 7.2, 150 mM NaCl, 0.1 % Triton X-100

20 9. Block buffer: TBST, 0.1 % ethanolamine pH 7.4

10. DMSO, Sigma, St. Louis, MO

11. Kinase buffer (KB): 20 mM HEPES/HCl pH 7.2, 150 mM NaCl, 0.1 % Triton X-100, 1 mM PMSF, 5 mg/L Aprotinin, 75 mM sodium ortho vanadate, 0.5 mM DTT and 10 mM MgCl₂.

12. ATP mix: 100 mM MgCl₂, 300 mM ATP, 10 mCi ³³P ATP
(Dupont-NEN) /mL.

13 Stop solution: 1 % phosphoric acid; Fisher,
Pittsburgh, PA.

5 14. Wallac Cellulose Phosphate Filter mats; Wallac,
Turku, Finnland.

15. Filter wash solution: 1 % phosphoric acid, Fisher,
Pittsburgh, PA.

16. Tomtec plate harvester, Wallac, Turku, Finnland.

10 17. Wallac beta plate reader # 1205, Wallac, Turku,
Finnland.

18. NUNC 96-well V bottom polypropylene plates for
compounds Applied Scientific Catalog # AS-72092.

15 Procedure

All of the following steps are conducted at room
temperature unless specifically indicated.

1. ELISA plate coating: ELISA wells are coated with 100
ml of Sheep anti mouse affinity purified antiserum (1 mg/100 mL
20 coating buffer) over night at 4 °C. ELISA plates can be used for
two weeks when stored at 4 °C.

2. Invert the plate and remove liquid. Add 100 mL of
blocking solution and incubate for 30 min.

3. Remove blocking solution and wash four times with

wash buffer. Pat the plate on a paper towel to remove excess liquid.

4. Add 1 mg of antibody specific for RAF-1 to each well and incubate for 1 hour. Wash as described in step 3.

5 5. Thaw lysates from RAS/RAF infected Sf9 cells and dilute with TBST to 10 mg/100 mL. Add 10 mg of diluted lysate to the wells and incubate for 1 hour. Shake the plate during incubation. Negative controls receive no lysate. Lysates from RAS/RAF infected Sf9 insect cells are prepared after cells are
10 infected with recombinant baculoviruses at a MOI of 5 for each virus, and harvested 48 hours later. The cells are washed once with PBS and lysed in RIPA buffer. Insoluble material is removed by centrifugation (5 min at 10 000 x g). Aliquots of lysates are frozen in dry ice/ethanol and stored at -80 °C until use.

15 6. Remove non-bound material and wash as outlined above (step 3).

7. Add 2 mg of T-MEK and 2 mg of His-MAEPK per well and adjust the volume to 40 mL with kinase buffer. Methods for purifying T-MEK and MAPK from cell extracts are provided herein
20 by example.

8. Pre-dilute compounds (stock solution 10 mg/mL DMSO) or extracts 20 fold in TBST plus 1% DMSO. Add 5 mL of the pre-diluted compounds/extracts to the wells described in step 6. Incubate for 20 min. Controls receive no drug.

9. Start the kinase reaction by addition of 5 mL ATP mix; Shake the plates on an ELISA plate shaker during incubation.

10. Stop the kinase reaction after 60 min by addition of 30 mL stop solution to each well.

5 11. Place the phosphocellulose mat and the ELISA plate in the Tomtec plate harvester. Harvest and wash the filter with the filter wash solution according to the manufacturers recommendation. Dry the filter mats. Seal the filter mats and place them in the holder. Insert the holder into radioactive
10 detection apparatus and quantify the radioactive phosphorous on the filter mats.

Alternatively, 40 mL aliquots from individual wells of the assay plate can be transferred to the corresponding positions on the phosphocellulose filter mat. After air drying the
15 filters, put the filters in a tray. Gently rock the tray, changing the wash solution at 15 min intervals for 1 hour. Air-dry the filter mats. Seal the filter mats and place them in a holder suitable for measuring the radioactive phosphorous in the samples. Insert the holder into a detection device and quantify
20 the radioactive phosphorous on the filter mats.

j. CDK2/Cyclin A - Inhibition Assay

This assay analyzes the protein kinase activity of CDK2 in exogenous substrate.

Reagents:

- A. Buffer A (80 mM Tris (pH 7.2), 40 mM MgCl₂): 4.84 G.
Tris (F.W. =121.1 g/mol), 4.07 g. MgCl₂ (F.W.=203.31 g/mol) dissolved in 500 ml H₂O. Adjust pH to 7.2 with HCl.
- 5 B. Histone H1 solution (0.45 mg/ml Histone H1 and 20 mM HEPES pH 7.2 (pH 7.4 is OK): 5 mg Histone H1 (Boehinger Mannheim) in 11.111 ml 20 mM HEPES pH 7.2 (477 mg HEPES (F.W.= 238.3 g/mol) dissolved in 100 ml ddH₂O, stored in 1 ml aliquots at -80° C.
- 10 C. ATP solution (60 μM ATP, 300 μg/ml BSA, 3 mM DTT): 120 μl 10 mM ATP, 600 μl 10 mg/ml BSA to 20 ml, stored in 1 ml aliquots at -80° C.
- D. CDK2 solution: cdk2/cyclin A in 10 mM HEPES pH 7.2, 25 mM NaCl, 0.5 mM DTT, 10% glycerol, stored in 9 μl
- 15 aliquots at -80° C.

Description of Assay:

1. Prepare solutions of inhibitors at three times the desired final assay concentration in ddH₂O/15 % DMSO by volume.
- 20 2. Dispense 20 μl of inhibitors to wells of polypropylene 96-well plates (or 20 μl 15% DMSO for positive and negative controls).
3. Thaw Histone H1 solution (1 ml/plate), ATP solution (1

ml/plate plus 1 aliquot for negative control), and CDK2 solution (9 μ l/plate). Keep CDK2 on ice until use. Aliquot CDK2 solution appropriately to avoid repeated freeze-thaw cycles.

- 5 4. Dilute 9 μ l CDK2 solution into 2.1 ml Buffer A (per plate). Mix. Dispense 20 μ l into each well.
5. Mix 1 ml Histone H1 solution with 1 ml ATP solution (per plate) into a 10 ml screw cap tube. Add γ^{33} P ATP to a concentration of 0.15 μ Ci/20 μ l (0.15 μ Ci/well in assay).
- 10 Mix carefully to avoid BSA frothing. Add 20 μ l to appropriate wells. Mix plates on plate shaker. For negative control, mix ATP solution with an equal amount of 20 mM HEPES pH 7.2 and add γ^{33} P ATP to a concentration of 0.15 μ Ci/20 μ l solution. Add 20 μ l to appropriate
- 15 wells.
6. Let reactions proceed for 60 minutes.
7. Add 35 μ l 10% TCA to each well. Mix plates on plate shaker.
8. Spot 40 μ l of each sample onto P30 filter mat squares.
- 20 Allow mats to dry (approx. 10-20 minutes).
- 9 Wash filter mats 4 X 10 minutes with 250 ml 1% phosphoric acid (10 ml phosphoric acid per liter ddH₂O).
10. Count filter mats with beta plate reader.

2. Cellular/Biologic Assays

Assay 1: PDGF-Induced BrdU Incorporation

Assay

Materials and Reagents:

- 5 (1) PDGF: human PDGF B/B; 1276-956, Boehringer Mannheim,
Germany.
- (2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat.
No. 1 647 229, Boehringer Mannheim, Germany.
- 10 (3) FixDenat: fixation solution (ready to use), Cat. No.
1 647 229, Boehringer Mannheim, Germany.
- (4) Anti-BrdU-POD: mouse monoclonal antibody conjugated
with peroxidase, Cat. No. 1 647 229, Boehringer
Mannheim, Germany.
- 15 (5) TMB Substrate Solution: tetramethylbenzidine (TMB),
ready to use, Cat. No. 1 647 229, Boehringer
Mannheim, Germany.
- (6) PBS Washing Solution : 1X PBS, pH 7.4, made in house
(Sugen, Inc., Redwood City, California).
- 20 (7) Albumin, Bovine (BSA): fraction V powder; A-8551,
Sigma Chemical Co., USA.
- (8) 3T3 cell line genetically engineered to express
human PDGF-R.

Protocol

- (1) Cells are seeded at 8000 cells/well in DMEM, 10% CS, 2mM Gln in a 96 well plate. Cells are incubated overnight at 37°C in 5% CO₂.
- (2) After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0%CS DMEM with 0.1% BSA) for 24 hours.
- (3) On day 3, ligand (PDGF, 3.8 nM, prepared in DMEM with 0.1% BSA) and test compounds are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand (PDGF) but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.
- (4) After 20 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 µM) for 1.5 hours.
- (5) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 µl/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.
- (6) The FixDenat solution is thoroughly removed by

decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 μ l/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.

- (7) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 μ l/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.
- (8) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.
- (9) TMB substrate solution is added (100 μ l/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.
- (10) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

Assay 2: EGF-Induced BrdU Incorporation

Assay

Materials and Reagents

- (1) EGF: mouse EGF, 201; Toyobo, Co., Ltd. Japan.
- (2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat. No. 1 647 229, Boehringer Mannheim, Germany.
- 5 (3) FixDenat: fixation solution (ready to use), Cat. No. 1 647 229, Boehringer Mannheim, Germany.
- (4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.
- 10 (5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.
- (6) PBS Washing Solution : 1X PBS, pH 7.4, made in house (Sugen, Inc., Redwood City, California).
- 15 (7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.
- (8) 3T3 cell line genetically engineered to express human EGF-R.

20

Protocol

- (1) Cells are seeded at 8000 cells/well in 10% CS, 2mM Gln in DMEM, in a 96 well plate. Cells are incubated overnight at 37°C in 5% CO₂.

- (2) After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0% CS DMEM with 0.1% BSA) for 24 hours.
- (3) On day 3, ligand (EGF, 2 nM, prepared in DMEM with 0.1% BSA) and test compounds are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand (EGF) but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.
- (4) After 20 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 μ M) for 1.5 hours.
- (5) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 μ l/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.
- (6) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 μ l/well) as a blocking solution and the plate is

incubated for 30 minutes at room temperature on a plate shaker.

- 5 (7) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 μ l/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.
- 10 (8) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.
- 15 (9) TMB substrate solution is added (100 μ l/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.
- (10) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

20 **Assay 3: EGF-Induced Her2-Driven BrdU
Incorporation**

Materials and Reagents:

- (1) EGF: mouse EGF, 201; Toyobo, Co., Ltd. Japan
- (2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat.

No. 1 647 229, Boehringer Mannheim, Germany.

(3) FixDenat: fixation solution (ready to use), Cat. No.
1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated
with peroxidase, Cat. No. 1 647 229, Boehringer
Mannheim, Germany.

(5) TMB Substrate Solution: tetramethylbenzidine (TMB),
ready to use, Cat. No. 1 647 229, Boehringer
Mannheim, Germany.

(6) PBS Washing Solution: 1X PBS, pH 7.4, made in house.

(7) Albumin, Bovine (BSA): fraction V powder; A-8551,
Sigma Chemical Co., USA.

(8) 3T3 cell line engineered to express a chimeric
receptor having the extra-cellular domain of EGF-R
and the intra-cellular domain of Her2.

Protocol:

(1) Cells are seeded at 8000 cells/well in DMEM, 10% CS,
2mM Gln in a 96- well plate. Cells are incubated
overnight at 37° in 5% CO₂.

(2) After 24 hours, the cells are washed with PBS, and
then are serum starved in serum free medium (0%CS
DMEM with 0.1% BSA) for 24 hours.

(3) On day 3, ligand (EGF=2 nM, prepared in DMEM with
0.1% BSA) and test compounds are added to the cells

simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand (EGF) but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

(4) After 20 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration = 10 μ M) for 1.5 hours.

(5) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 μ l/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.

(6) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 μ l/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.

(7) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added

(100 μ l/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.

(8) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.

(9) TMB substrate solution is added (100 μ l/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.

(10) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

Assay 4: IGF1-Induced BrdU Incorporation

Assay

Materials and Reagents:

- (1) IGF1 Ligand: human, recombinant; G511, Promega Corp, USA.
- (2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat. No. 1 647 229, Boehringer Mannheim, Germany.
- (3) FixDenat: fixation solution (ready to use), Cat. No.

1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

5 (5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(6) PBS Washing Solution: 1X PBS, pH 7.4, made in house (Sugen, Inc., Redwood City, California).

10 (7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.

(8) 3T3 cell line genetically engineered to express human IGF-1 receptor.

Protocol:

15 (1) Cells are seeded at 8000 cells/well in DMEM, 10% CS, 2mM Gln in a 96- well plate. Cells are incubated overnight at 37°C in 5% CO₂.

(2) After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0%CS
20 DMEM with 0.1% BSA) for 24 hours.

(3) On day 3, ligand (IGF1=3.3 nM, prepared in DMEM with 0.1% BSA) and test compounds are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive

control cells receive the ligand (IGF1) but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

- 5 (4) After 16 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 μ M) for 1.5 hours.
- 10 (5) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 μ l/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.
- 15 (6) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 μ l/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.
- 20 (7) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 μ l/well) and the plate is incubated for 90

minutes at room temperature on a plate shaker.

- (8) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.

- (9) TMB substrate solution is added (100 μ l/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.

- (10) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

g. HUV-EC-C Assay

The following protocol may also be used to measure a compound's activity against PDGF-R, FGF-R, VEGF, aFGF or Flk-1/KDR, all of which are naturally expressed by HUV-EC cells.

DAY 0

1. Wash and trypsinize HUV-EC-C cells (human umbilical vein endothelial cells, (American Type Culture Collection; catalogue no. 1730 CRL). Wash with Dulbecco's phosphate-buffered

saline (D-PBS; obtained from Gibco BRL; catalogue no. 14190-029)
2 times at about 1 ml/10 cm² of tissue culture flask. Trypsinize
with 0.05% trypsin-EDTA in non-enzymatic cell dissociation
solution (Sigma Chemical Company; catalogue no. C-1544). The
5 0.05% trypsin is made by diluting 0.25% trypsin/1 mM EDTA (Gibco;
catalogue no. 25200-049) in the cell dissociation solution.
Trypsinize with about 1 ml/25-30 cm² of tissue culture flask for
about 5 minutes at 37°C. After cells have detached from the
flask, add an equal volume of assay medium and transfer to a 50
10 ml sterile centrifuge tube (Fisher Scientific; catalogue no. 05-
539-6).

2. Wash the cells with about 35 ml assay medium in the
50 ml sterile centrifuge tube by adding the assay medium,
centrifuge for 10 minutes at approximately 200 g, aspirate the
15 supernatant, and resuspend with 35 ml D-PBS. Repeat the wash two
more times with D-PBS, resuspend the cells in about 1 ml assay
medium/15 cm² of tissue culture flask. Assay medium consists of
F12K medium (Gibco BRL; catalogue no. 21127-014) + 0.5% heat-
inactivated fetal bovine serum. Count the cells with a Coulter
20 Counter, Coulter Electronics, Inc.) and add assay medium to the
cells to obtain a concentration of 0.8-1.0x10⁵ cells/ml.

3. Add cells to 96-well flat-bottom plates at 100
µl/well or 0.8-1.0x10⁴ cells/well; incubate -24h at 37°C, 5% CO₂.

DAY 1

1. Make up two-fold drug titrations in separate 96-well plates, generally 50 μ M on down to 0 μ M. Use the same assay medium as mentioned in day 0, step 2 above. Titrations are made by adding 90 μ l/well of drug at 200 μ M (4X the final well concentration) to the top well of a particular plate column. Since the stock drug concentration is usually 20 mM in DMSO, the 200 μ M drug concentration contains 2% DMSO.

Therefore, diluent made up to 2% DMSO in assay medium (F12K + 0.5% fetal bovine serum) is used as diluent for the drug titrations in order to dilute the drug but keep the DMSO concentration constant. Add this diluent to the remaining wells in the column at 60 μ l/well. Take 60 μ l from the 120 μ l of 200 μ M drug dilution in the top well of the column and mix with the 60 μ l in the second well of the column. Take 60 μ l from this well and mix with the 60 μ l in the third well of the column, and so on until two-fold titrations are completed. When the next-to-the-last well is mixed, take 60 μ l of the 120 μ l in this well and discard it. Leave the last well with 60 μ l of DMSO/media diluent as a non-drug-containing control. Make 9 columns of titrated drug, enough for triplicate wells each for 1) VEGF (obtained from Pepro Tech Inc., catalogue no. 100-200, 2) endothelial cell growth factor (ECGF) (also known as acidic fibroblast growth factor, or aFGF) (obtained from Boehringer Mannheim Biochemica,

catalogue no. 1439 600); or, 3) human PDGF B/B (1276-956, Boehringer Mannheim, Germany) and assay media control. ECGF comes as a preparation with sodium heparin.

2. Transfer 50 μ l/well of the drug dilutions to the 96-well assay plates containing the $0.8-1.0 \times 10^4$ cells/100 μ l/well of the HUV-EC-C cells from day 0 and incubate ~2 h at 37°C, 5% CO₂.

3. In triplicate, add 50 μ l/well of 80 μ g/ml VEGF, 20 ng/ml ECGF, or media control to each drug condition. As with the drugs, the growth factor concentrations are 4X the desired final concentration. Use the assay media from day 0 step 2 to make the concentrations of growth factors. Incubate approximately 24 hours at 37°C, 5% CO₂. Each well will have 50 μ l drug dilution, 50 μ l growth factor or media, and 100 μ l cells, = 200 μ l/well total. Thus the 4X concentrations of drugs and growth factors become 1X once everything has been added to the wells.

DAY 2

1. Add ³H-thymidine (Amersham; catalogue no. TRK-686) at 1 μ Ci/well (10 μ l/well of 100 μ Ci/ml solution made up in RPMI media + 10% heat-inactivated fetal bovine serum) and incubate ~24 h at 37°C, 5% CO₂. RPMI is obtained from Gibco BRL, catalogue no. 11875-051.

DAY 3

1. Freeze plates overnight at -20°C.

DAY 4

1. Thaw plates and harvest with a 96-well plate harvester (Tomtec Harvester 96^(R)) onto filter mats (Wallac; catalogue no. 1205-401); read counts on a Wallac Betaplate^(TM) liquid scintillation counter.

3. *In Vivo* Animal Models

A. Xenograft Animal Models

The ability of human tumors to grow as xenografts in athymic mice (e.g., Balb/c, nu/nu) provides a useful in vivo model for studying the biological response to therapies for human tumors. Since the first successful xenotransplantation of human tumors into athymic mice, (Rygaard and Povlsen, 1969, Acta Pathol. Microbial. Scand. 77:758-760), many different human tumor cell lines (e.g., mammary, lung, genitourinary, gastro-intestinal, head and neck, glioblastoma, bone, and malignant melanomas) have been transplanted and successfully grown in nude mice. The following assays may be used to determine the level of activity, specificity and effect of the different compounds of the present invention. Three general types of assays are useful for evaluating compounds: cellular/catalytic, cellular/biological and in vivo. The object of the cellular/catalytic assays is to determine the effect of a compound on the ability of a TK to phosphorylate tyrosines on a known substrate in a cell. The object of the cellular/biological

assays is to determine the effect of a compound on the biological response stimulated by a TK in a cell. The object of the in vivo assays is to determine the effect of a compound in an animal model of a particular disorder such as cancer.

5 Suitable cell lines for subcutaneous xenograft experiments include C6 cells (glioma, ATCC # CCL 107), A375 cells (melanoma, ATCC # CRL 1619), A431 cells (epidermoid carcinoma, ATCC # CRL 1555), Calu 6 cells (lung, ATCC # HTB 56), PC3 cells (prostate, ATCC # CRL 1435), SKOV3TP5 cells and NIH 3T3
10 fibroblasts genetically engineered to overexpress EGFR, PDGFR, IGF-1R or any other test kinase. The following protocol can be used to perform xenograft experiments:

Female athymic mice (BALB/c, nu/nu) are obtained from Simonsen Laboratories (Gilroy, CA). All animals are maintained
15 under clean-room conditions in Micro-isolator cages with Alpha-dri bedding. They receive sterile rodent chow and water ad libitum.

Cell lines are grown in appropriate medium (for example, MEM, DMEM, Ham's F10, or Ham's F12 plus 5% - 10% fetal
20 bovine serum (FBS) and 2 mM glutamine (GLN)). All cell culture media, glutamine, and fetal bovine serum are purchased from Gibco Life Technologies (Grand Island, NY) unless otherwise specified. All cells are grown in a humid atmosphere of 90-95% air and 5-10% CO₂ at 37°C. All cell lines are routinely

subcultured twice a week and are negative for mycoplasma as determined by the Mycotect method (Gibco).

Cells are harvested at or near confluency with 0.05% Trypsin-EDTA and pelleted at 450 x g for 10 min. Pellets are
5 resuspended in sterile PBS or media (without FBS) to a particular concentration and the cells are implanted into the hindflank of the mice (8 - 10 mice per group, $2 - 10 \times 10^6$ cells/animal). Tumor growth is measured over 3 to 6 weeks using venier calipers. Tumor volumes are calculated as a
10 product of length x width x height unless otherwise indicated. P values are calculated using the Students t-test. Test compounds in 50 - 100 μ L excipient (DMSO, or VPD:D5W) can be delivered by IP injection at different concentrations generally starting at day one after implantation.

15 **B. Tumor Invasion Model**

The following tumor invasion model has been developed and may be used for the evaluation of therapeutic value and efficacy of the compounds identified to selectively inhibit KDR/FLK-1 receptor.

20 **Procedure**

8 week old nude mice (female) (Simonsen Inc.) are used as experimental animals. Implantation of tumor cells can be performed in a laminar flow hood. For anesthesia, Xylazine/Ketamine Cocktail (100 mg/kg ketamine and 5 mg/kg

Xylazine) are administered intraperitoneally. A midline incision is done to expose the abdominal cavity (approximately 1.5 cm in length) to inject 10^7 tumor cells in a volume of 100 μ l medium. The cells are injected either into the duodenal lobe of the pancreas or under the serosa of the colon. The peritoneum and muscles are closed with a 6-0 silk continuous suture and the skin is closed by using wound clips. Animals are observed daily.

Analysis

After 2-6 weeks, depending on gross observations of the animals, the mice are sacrificed, and the local tumor metastases in various organs (lung, liver, brain, stomach, spleen, heart, muscle) are excised and analyzed (measurement of tumor size, grade of invasion, immunochemistry, in situ hybridization, etc.).

D. Measurement Of Cell Toxicity

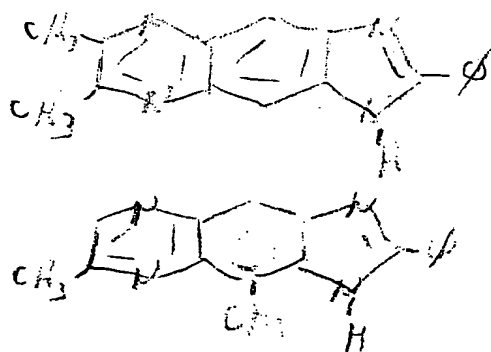
Therapeutic compounds should be more potent in inhibiting receptor tyrosine kinase activity than in exerting a cytotoxic effect. A measure of the effectiveness and cell toxicity of a compound can be obtained by determining the therapeutic index: IC_{50}/LD_{50} . IC_{50} , the dose required to achieve 50% inhibition, can be measured using standard techniques such as those described herein. LD_{50} , the dosage which results in 50% toxicity, can also be measured by standard techniques (Mossman,

1983, J. Immunol. Methods, 65:55-63), by measuring the amount of LDH released (Korzeniewski and Callewaert, 1983, J. Immunol. Methods, 64:313; Decker and Lohmann-Matthes, 1988, J. Immunol. Methods, 115:61), or by measuring the lethal dose in animal models. Compounds with a large therapeutic index are preferred. The therapeutic index should be greater than 2, preferably at least 10, more preferably at least 50.

TABLE 3

RESULTS OF ASSAYS OF SELECTED COMPOUNDS

NAME	PDGFR KINASE IC50 (μ M)	FLK KINASE IC50 (μ M)	EGFR KINASE IC50 (μ M)
5,6,-dimethyl-2-phenylimidazo[4,5- g]quinoxaline	1.9	>10	>10
6,7,-dimethyl-2-phenylimidazo[4,5- g]quinoxaline	4.3	88.4	>100



CONCLUSION

Thus, it will be appreciated that the compounds, methods and pharmacological compositions of the present invention are expected to modulate RTK and CTK activity and therefore to be effective as therapeutic agents against RTK- and CTK-related disorders.

Although certain embodiments and examples have been used to describe the present invention, it will be apparent to those skilled in the art that changes to the embodiments and examples shown may be made without departing from the scope and spirit of the invention.

Other embodiments are within the following claims.